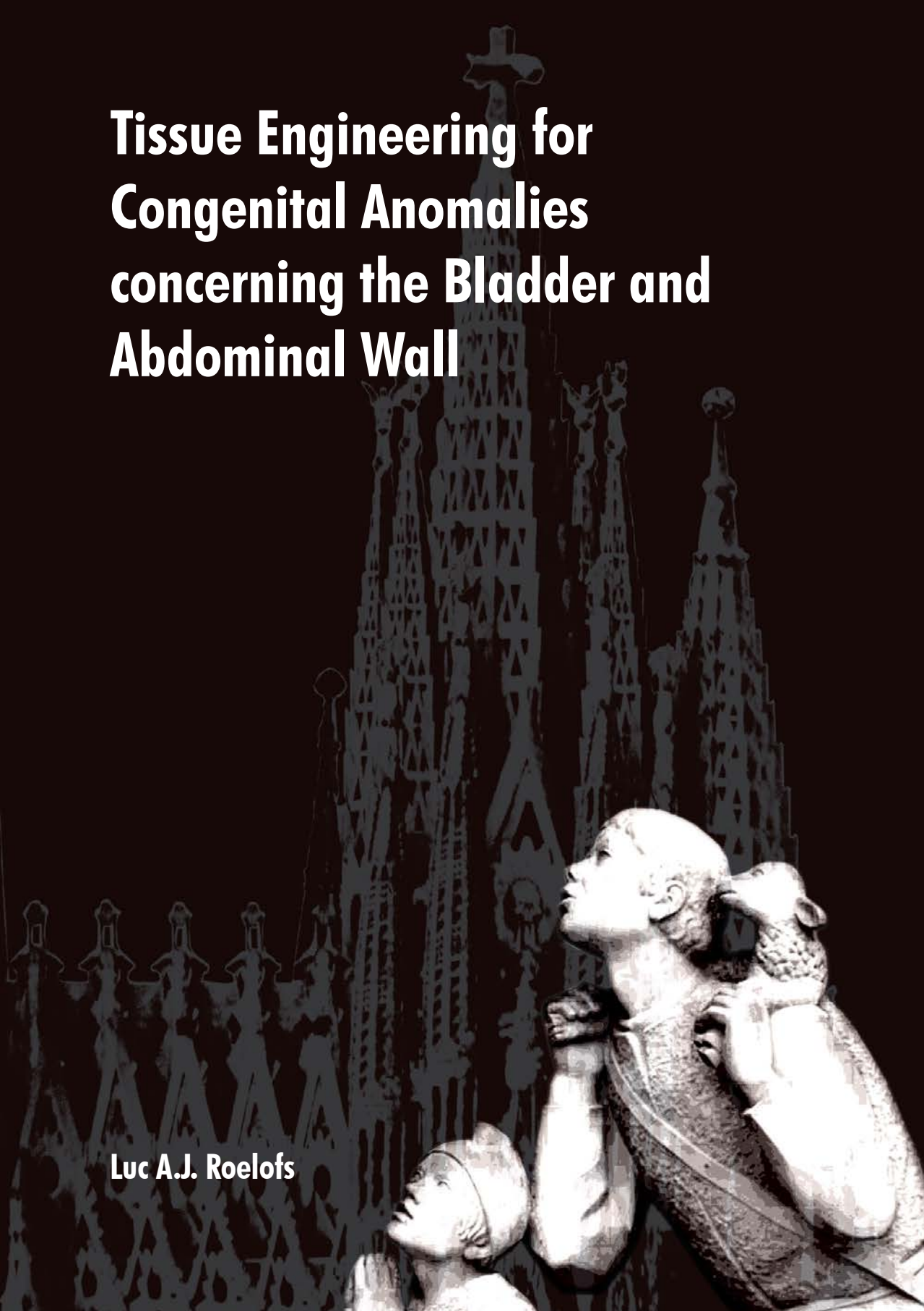
Tissue Engineering for Congenital Anomalies concerning the Bladder and Abdominal Wall



Luc A.J. Roelofs

Tissue Engineering for Congenital Anomalies concerning the Bladder and Abdominal Wall

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The studies presented in this thesis were performed at the Department of Urology, Nijmegen Centre for Molecular Life Sciences (NCMLS), Radboud university medical center, Nijmegen, the Netherlands. This thesis was financially supported by 'Vrije beleidsruimte 2003' OZ-2003-1 from Radboud university medical center, the Netherlands, FP6-project EuroSTEC, contract LSHB-CT-2006-037409 from the European Union, and 'AGIKO stipendium' project number 920-03-456 from 'the Netherlands Organisation for Health Research and Development' (ZonMw) and Radboud university medical center, the Netherlands.

Publication of this thesis was financially supported by:
Astellas Pharma B.V., Hoogland Medical, Abbvie B.V., Allergan B.V., Amgen B.V., Covidien, De Bedrijfspoli, Ferring B.V., GlaxoSmithKline, Janssen-Cilag B.V., Medical Measurement Systems B.V., Olympus Nederland B.V., Pfizer B.V., Porgès a Coloplast Division, Takeda Nederland B.V., Wellspect Healthcare, Zambon Nederland B.V.

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ISBN: 978-94-6108-646-4
Cover design and lay-out: Martijn van Leipsig, Utrecht, the Netherlands
Photography: L.A.J. Roelofs, Nijmegen, the Netherlands
Printed by: Gildeprint, Enschede, the Netherlands

Tissue Engineering for Congenital Anomalies concerning the Bladder and Abdominal Wall

Proefschrift

ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de rector magnificus prof. mr. S.C.J.J. Kortmann,
volgens besluit van het College van Decanen
in het openbaar te verdedigen op 11 juli 2014
om 10.30 uur precies

door

Lucas Antoon Jacco Roelofs

geboren op 14 april 1978
te Ewijk

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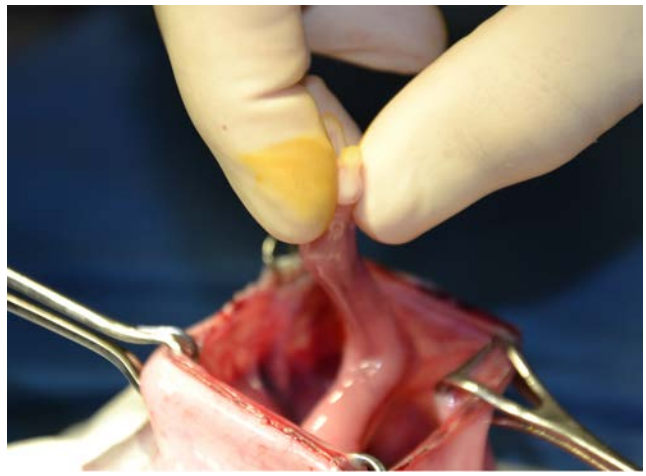
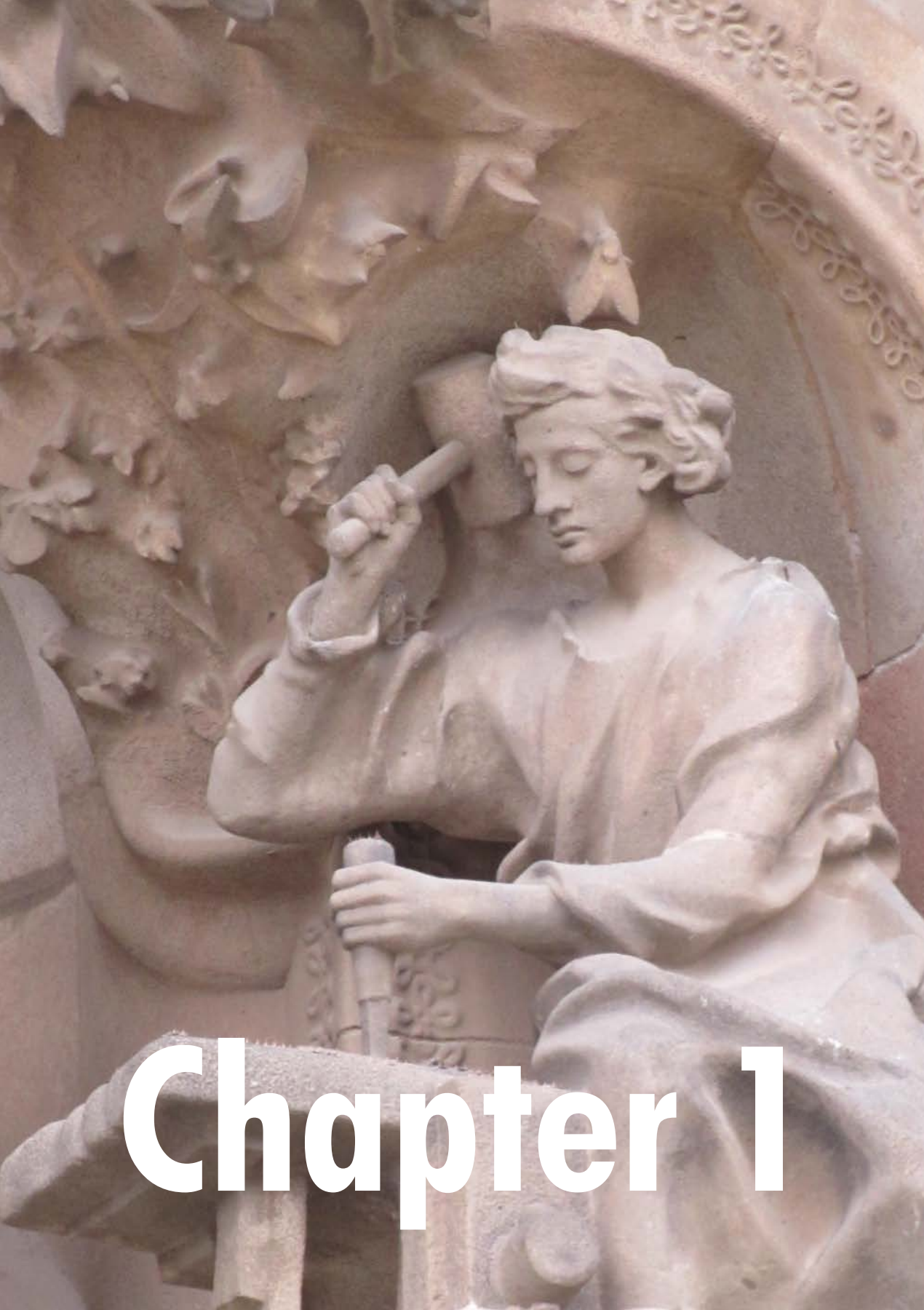


Foto gemaakt tijdens een foetale operatie aan een lam voor het onderzoek verricht in dit proefschrift. De foto verwijst naar de foto genaamd: "Hand of Hope", welke op 19 augustus 1999 werd gemaakt door fotograaf Michael Clancy. De betreffende foto werd genomen toen Dr. Joseph Bruner en collega Dr. Noel Tulipan in het ziekenhuis Vanderbilt University Nashville USA de foetus Samuel Armas opereerden bij een zwangerschapsduur van 21 weken. Bij Samuel Armas was een spina bifida gediagnosticeerd. Tijdens de foetale operatie werd het blootliggende ruggenmerg bedekt, om verdere secundaire schade te voorkomen, zodat het kind na de geboorte minder morbiditeit zou ondervinden. Op de foto is zichtbaar dat de foetale hand van Samuel Armas een vinger grijpt van Dr. Bruner, alsof deze de arts wil vragen hem te helpen.
Zie: http://en.wikipedia.org/wiki/Samuel_Armas en http://michaelclancy.com/

In 2011 werd in de New England Journal of Medicine de 'MOMS-trial' (Management Of Myelomeningocele Study) gepubliceerd, waarin werd aangetoond dat kinderen waarbij middels een foetale operatie het ruggenmerg werd bedekt minder morbiditeit hadden dan kinderen met een conventionele postnatale operatie.
Zie: Adzick NS et al. A randomized trial of prenatal versus postnatal repair of myelomeningocele. N Engl J Med 2011;364:993-1004.

Voor mijn ouders



Chapter 1

General Introduction and Outline of this Thesis

Introduction

In this thesis, innovative personal health care developments in the field of regenerative medicine for children with a congenital anomaly are described. In the first part of this thesis, tissue engineering techniques for children with a dysfunctioning bladder are discussed. In the second part, tissue engineering of the abdominal wall for children with abdominal wall defects such as gastroschisis are discussed.
In this introduction, we describe the congenital anomalies and the clinical problems of patients with these anomalies, which may benefit from the new tissue engineering techniques investigated in this thesis. Furthermore, we describe the basic principles of tissue engineering, and the current knowledge of tissue engineering performed for the involved anomalies. Finally, the aims of this thesis are presented in the section 'Outline of this thesis'.

Congenital anomalies which may need bladder augmentation
The bladder serves as a reservoir for urine. During the storage phase urine of an appropriate volume is stored without leakage, while maintaining low pressure in the bladder. Voiding is under voluntary control, and during the voiding phase the bladder is completely emptied, after descent of the bladder neck, contraction of the detrusor muscle, reflexive opening of the bladder neck and sequential opening of the external urinary sphincter [1]. Normally the bladder is elastic and highly compliant, resulting in low pressures in the bladder during the filling phase. Elevated passive filling pressure becomes problematic when chronically above 40 cmH_2O, hereby hampering adequate ureteral drainage resulting in damage to the kidneys [1]. Children with congenital anomalies such as ***bladder exstrophy, myelomeningocele*** or ***posterior urethral valves*** can develop small-capacity bladders with low compliance and high intravesical pressures. Bladder augmentation may be needed when drug therapy and/or intermittent catheterization fails, to create adequate reservoir function and maintain a low intravesical pressure to preserve upper urinary tract function [1].

Bladder exstrophy occurs in approximately between 1 in 10,000 and 1 in 50,000 live births [2]; and 0.5 in 10,000 live births in the Netherlands according to the EUROCAT Update 1981-2011 of Northern Netherlands [3]. This anomaly is thought to be caused by the rupture of the cloacal membrane that divides the fetal cloaca into a bladder anteriorly and a rectum posteriorly [4]. Genetic studies are currently performed to investigate the underlying cause. The rupture may cause bladder exstrophy, epispadias or cloacal exstrophy (the most severe form of the exstrophy-epispadias complex), depending on the extent of the infraumbilical defect and the stage of development during which it occurs. In bladder exstrophy this results in an open bladder plate and bladder neck, and epispadias. The exstrophied bladder is

situated in a closure defect of the lower abdominal wall (Figure 1).
At birth, the bladder mucosa may appear normal, but polyps may be present, and cystic or metaplastic changes may occur in the absence of frequent irrigation and a protective membrane [5]. Furthermore, increased collagen content results in fibrosis of the bladder plate [6,7], and the number of small nerve fibres is reduced [8]. At birth, the exstrophied bladder is thought to be in an earlier developmental stage compared to normal bladders [5]. Tissue samples of neonates and older patients reveal severe histological abnormalities, even after primary or delayed closure of the bladder [9-11]. Acute or chronic inflammation can be found, with ulceration and squamous metaplasia, cystitis cystica and cystitis glandularis. In the submucosa, fibrosis and dilated vessels are present. In the detrusor muscle, fibrosis and disorganization of the muscle arrangement may occur.

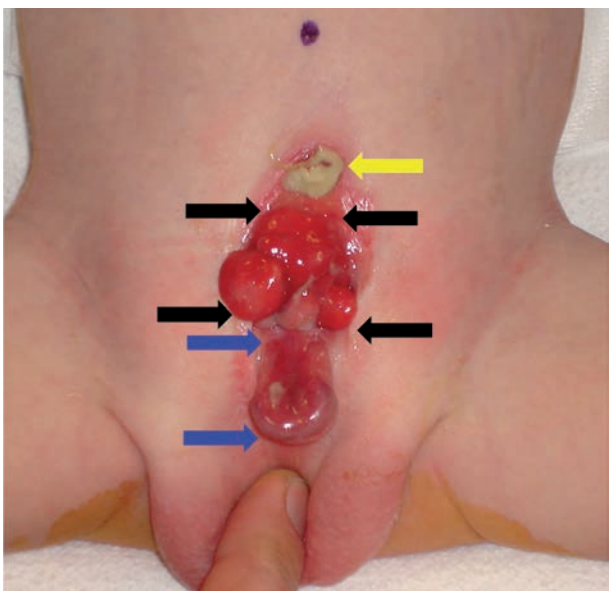


Figure 1. *Male patient with bladder exstrophy, after birth. Open bladder plate, covered with polyps, between black arrows. Umbilicus at yellow arrow. Penis with epispadias at blue arrows.*

Reconstruction is generally performed within days after birth. In the first stage a bilateral iliac osteotomy is mostly performed, the bladder and abdominal wall are closed, and a posterior urethral closure well onto the penis can be performed. Epispadias correction can be performed directly or at 6-12 months of age. When the child has achieved adequate bladder capacity for bladder neck reconstruction and is motivated to participate in a postoperative voiding program, approximately at the age of 4-5 years a bladder neck reconstruction and an antireflux procedure is performed [5]. Functional repair may be impossible when the bladder is small,

fibrotic, inelastic, and covered with polyps, in which case the operation can be postponed until the bladder plate has reached sufficient size [5].
In some patients the bladder will not reach sufficient capacity or compliance after bladder neck closure. Bladder augmentation will be required in these cases [5].

Myelomeningocele accounts for more than 90% of open myelodysplastic lesions [12] of which the incidence is about 4.1 per 10,000 live births in the Netherlands according to the EUROCAT Update 1981-2011 of Northern Netherlands [3]. Failure of fusion of the neural tube in the caudal region leads to spina bifida. Bone and muscle are unable to grow over the open section of the neural tube, resulting in a defect through which the spinal cord, nerves and meninges protrude [13].
The severity of symptoms is depending on the level of the defect and can result in paralysis of the legs, bowel and bladder dysfunction, sensibility disorders of the skin, sexual dysfunction and deformation of the lower extremities and back. Abnormalities on urodynamic testing are seen in more than 90% of neonates [14]. Urodynamics show bladder contractions in 63% of patients, of whom 50% have detrusor overactivity; 46% of those with no contractions have poor bladder compliance [15,16]. The external urethral sphincter innervation is intact in 40%, partially intact in 24%, and denervation is seen in 36%. This results in a frequently used classification of bladder and sphincter function: synergic (26%); dyssynergic with and without poor detrusor compliance (37%); and complete denervation (36%) [16,17]. Particularly the dyssynergic group is at risk of developing a poorly compliant bladder with high intravesical pressure. Of this group, 71% will have urinary tract deterioration within 3 years [18]. Intermittent catheterization alone or in combination with anticholinergic agents decreases risk of deterioration to 8-10% [19,20], and botulinum-A toxin injections in the detrusor muscle can be used in nonresponders [21]. Bladder augmentation or urinary diversion is needed when this policy fails [12].

Posterior urethral valves occur in 1 in 8,000 to 25,000 live births [22,23].Children with this anomaly have a membrane in the urethra, originating from the verumontanum, which ends at the anterior part of the urethra. The embryology may be related to an abnormal insertion of the mesonephric ducts into the fetal cloaca [24]. The level of damage caused by these posterior urethral valves (PUV) depends on the degree of obstruction and duration of existence. PUV can result in high-pressure urine storage and voiding affecting the proximal urethra, prostate, bladder neck, bladder, ureters and kidneys. The bladder can show hypertrophy and hyperplasia of the detrusor muscle, resulting in poor compliance. However, severe obstruction can result in oligohydramnios, pulmonary hypoplasia, severe kidney failure, and even fetal death [24]. After birth, the valves are ablated and bladder function generally improves. However, in some boys bladder recovery is not adequate to protect the upper urinary tracts or the boy remains incontinent, in which cases catheterization

and anticholinergics are started [24]. When this therapy fails, bladder augmentation may be indicated [25,26].

Bladder augmentation

Currently, bladder augmentation is usually performed with the use of gastrointestinal tissue. Generally, a piece of about 20-40 cm of distal ileum, depending on the patient's size, native bladder capacity and desired final capacity, is detubularized and reconfigured to a spherical shape. The bowel is sutured to the bladder which is incised and split into halves. Other segments of the gastrointestinal tract have been used as well, such as stomach, cecum, colon or sigmoid [1]. In our hospital, 41 pediatric patients underwent an enterocystoplasty between January 1983 and June 2006. The underlying conditions were neuropathic bladder (63%), bladder exstrophy (27%) or posterior urethral valves (10%) [27]. However, the use of gastrointestinal tissue can lead to metabolic disturbances, infections, excessive mucus production, stone formation, perforation and even malignancies, and sufficient bowel tissue is not always available [1,28-30]. In our hospital complications were seen in 20 of 41 operated children (49%) [27]. Therefore, alternatives for the use of gastrointestinal tissues are desirable to decrease current complications.

Urinary diversion

A uretero-ileo-cutaneostomy can be indicated when reconstruction of a severely damaged bladder fails, or particularly after cystectomy in patients with bladder cancer. This incontinent conduit, or urostomy according to Bricker, has been the primary technique for incontinent urinary diversion for over 50 years [31]. However, the complication rate ranges between 18-56% within the first month after surgery [32-35]. Long-term complication rate rises to 45% after 5 years, and even 94% after 15 years [31]. Early complications are usually related to the bowel resection and anastomosis, i.e. anastomotic leakage, enteric fistula, bowel obstruction or prolonged ileus, whereas long-term complications are mainly stoma related [32]. Therefore, rather than using gastrointestinal tissue, it would be preferable to create a urinary conduit from an artificial construct through which urine can be diverted. Surgery time and bowel complications may be reduced, and hospitalisation may be shortened with such a procedure [36].

Abdominal wall defects: Gastroschisis

Gastroschisis is an abdominal wall defect that results in herniation of a large part of the bowel outside the abdominal cavity, where it is in direct contact with the amniotic fluid (Figure 2). The incidence seems to be increasing and is estimated to be 2.98 to 4.4 per 10,000 live births in the USA and Europe [37,38], and 0.6 per 10,000 live births in the Netherlands according to the EUROCAT Update 1981-2011 of Northern Netherlands [3]. The embryogenesis of gastroschisis is controversial. A number of theories have been proposed: a gestational vascular accident of

the omphalomesenteric vein causing necrosis of the abdominal wall; abnormal involution of the right umbilical vein; failure of differentiation of embryonic mesenchyme of the lateral folds of the body wall; and failure of the intestines to temporarily herniate in the umbilical stalk with rupture of the abdominal wall due to the rapidly increasing volume of the intestines [39,40].

Figure 2. *Patient with gastroschisis, after birth. Bowel loops are eviscerated.*

Mortality in neonates with gastroschisis is reported to be 4-12.5% [40-42]. Intra-uterine growth retardation and premature birth are frequently noted, and serious complications may occur, such as sepsis, bowel dysfunction, bowel atresia, and bowel necrosis with subsequent short bowel syndrome. Intestinal motility and absorption are decreased and postnatal feeding can be problematic [40-43]. At birth, the bowel is often covered with an inflammatory fibrous peel, and bowel loops are matted together, thickened, inflamed and edematous, and can be congested or ischemic. This damage to the eviscerated bowel may be the result of constriction at the site of the abdominal wall defect and/or the toxic effect of the amniotic fluid [44,45]. Damage to the bowel seems to occur during the last trimester of pregnancy, when the bowel is growing, hereby causing compression at the site of the abdominal wall defect. Additionally, the composition of amniotic fluid is changing due to the improving kidney function and the release of gastrointestinal waste products into the amniotic fluid [46-50].

Primary closure of the abdominal wall can be problematic because the abdominal cavity is relatively hypoplastic and the bowel volume is enlarged due to edema and fibrous peel formation (visceral-abdominal disproportion). Repositioning the bowel into the abdominal cavity will increase intra-abdominal pressure, and may result in respiratory problems, compromised venous blood flow, and abdominal compartment syndrome. In these cases a gradual abdominal wall closure with a

spring-loaded silo is often employed [51]. Occasionally prosthetic materials are needed to close the abdominal wall or the fascia defects. These materials, too, may cause complications, including wound infection, bowel fistula, erosion into abdominal viscera, lack of fixation, mesh extrusion, and extreme adhesion formation [52]. Furthermore, patch dehiscence may occur because the material does not grow with the child [53].
Gastroschisis can be detected in early pregnancy [54], which offers the opportunity to salvage the bowel tissue using fetal therapy. Tissue-engineered constructs could be a solution for the operative closure or coverage of these defects.

Tissue Engineering
Tissue engineering is part of the rapidly growing field of regenerative medicine and aims at repair or replacement of damaged or removed organs by inducing regenerative processes [55,56]. Constructs for tissue engineering should have the capacity to become structurally integrated with the surrounding tissue, and should initiate restoration of the essential functions of the lost or damaged target tissue [55,56]. These constructs consist of a carrier material forming the extracellular matrix (ECM), which serves as a 'skeleton' to support cell growth. Scaffolds can be prepared from natural (predominantly collagen) or synthetic polymers, or a combination of these. Collagen-based constructs can be composed of either decellularized tissue, e.g. bladder acellular matrix (BAM) [57] or small intestinal submucosa (SIS) [58,59], or of reconstituted purified collagen [60]. Collagen scaffolds can be combined with synthetic materials to enhance tensile strength of the construct or to decrease elasticity to protect the construct from collapsing [61,62]. After implantation, biodegradable constructs will be replaced by autologous ECM and cells. Prerequisites of these scaffold materials for tissue engineering include purity (to avoid immunological response due to contaminants e.g. cellular material), biocompatibility, biodegradability and good handling properties for the surgeon [55,56,60].
Collagen is a major component of the ECM, and type I collagen is the most abundant collagen in tissues like the bladder, skin, bone, and tendon [60]. The Department of Biochemistry at the Radboud university medical centre has developed methods to retrieve highly-purified type I collagen from bovine Achilles tendon [60,63], and to prepare scaffolds from this material. Structural properties of the scaffold are important. Cellular migration and supply of nutrients and oxygen is improved when the scaffold has a high porosity [60,64]. By freezing and lyophilisation techniques, a highly-porous collagen scaffold can be obtained [60,65]. Furthermore, decreasing the collagen content of the scaffolds improves interconnectivity of pores and cellular ingrowth [66]. To improve the strength of the scaffold, chemical crosslinking can be applied, e.g. using 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC). Crosslinking also prevents rapid degradation and diminishes antigenicity [60,63].

Scaffolds can be combined with autologous cells to improve regeneration and diminish scar tissue formation [61]. For this purpose cells need to be harvested from the target organ, expanded in culture flasks, seeded on the scaffolds, and cultured on the scaffolds prior to implantation. Different types of cells, and even combinations of cells, can be used depending on the tissue to be regenerated.
Growth factors are proteins involved in proliferation, migration and differentiation of several cell types, which makes them an interesting tool for tissue engineering [60,67-70]. Growth factors initiate their action by binding to specific receptors on target cells. Some growth factors can be coupled to scaffolds via heparin, a glycosaminoglycan (polysaccharide), that can be covalently bound to collagen scaffolds during the crosslinking process [60,67-70]. Growth factors are stabilised and protected from proteolytic degradation by their interaction with glycosaminoglycans [60]. A sustained release system is achieved by binding growth factors to a scaffold with heparin [67]. Combining different growth factors to a scaffold results in a 'smart' bioscaffold, which can modulate the regenerative process.

Tissue engineering of the bladder

During the last decade, *in vitro* and *in vivo* studies in various animal models have shown that new bladder tissue, including urothelium, smooth muscle, vessels and nerve fibres, can be regenerated with the use of tissue engineered constructs [71-75].
In the first clinical trial related to bladder tissue engineering, performed by Atala *et al.,* neuropathic bladders of 7 children with myelomeningocele were augmented [61]. Either a collagen scaffold or a composite of collagen and polyglycolic acid was used, and seeded with cultured autologous urothelial and smooth muscle cells. Particularly the composite scaffold wrapped in omentum, to support vascularisation, showed promising results. Subsequently, multi-centre phase II clinical studies were performed in pediatric patients with a neuropathic bladder due to spina bifida and in adult patients with spinal cord injury by Tengion, an American company involved in regenerative medicine [72,73,76-78]. These studies showed the feasibility of this technique. However, refinement is needed to further improve capacity and compliance before this technique can replace the current methods of bladder augmentation using gastro-intestinal tissue [61,72,73,76].
Preclinical animal studies have generally been performed in healthy bladders. However, translation to patients is hampered by observations that cultured urothelial and smooth muscle cells from patients with a neuropathic bladder or bladder exstrophy behave dissimilar from normal cells, which may have implications when used for tissue engineering purposes [79-85]. Indeed, two studies performed in an animal model for diseased bladder demonstrated that tissue engineering of diseased bladder led to much more fibrosis, fewer smooth muscle cells, and poor functionality [86,87]. Therefore, there is still a need for valid models to study tissue regeneration for congenital anomalies such as bladder exstrophy or myelomeningocele.

Cell-based constructs have been used to improve regeneration of bladder tissue, and to diminish fibrotic tissue formation and graft shrinkage [72-76]. Autologous urothelial and smooth muscle cells were harvested, cultured, and seeded on scaffolds. Cell-based constructs are thought to be necessary to create large constructs, which can lead to clinical significant improvement of bladder function [61,74,75]. However, this method is time consuming and expensive.

In large constructs, regeneration is hampered by the lack of oxygen and nutrition delivery to the cells, and by inadequate removal of waste products [68,88], since the amount of oxygen required for cell survival is limited to a diffusion distance of approximately 150-200 μm from the supplying blood vessel [88]. Therefore, angiogenesis needs to be improved, for which growth factors can be used. Growth factors are involved in proliferation, migration and differentiation of several cell types. Vascular endothelial growth factor 165 (VEGF165) is an important factor in angiogenesis. Especially in combination with fibroblast growth factor 2 (FGF2) it enhances blood vessel formation and maturation [68]. These two growth factors have been coupled to collagen scaffolds loaded with heparin, together with heparin-binding epidermal growth factor (HB-EGF), which is known to be involved in urothelial regeneration [69,89,90]. This smart bioscaffold was implanted in a rabbit urethra and the inclusion of growth factors had a profound positive effect on regenerative processes in this animal model [69]. Previous rat and rabbit studies in which a scaffold loaded with growth factors was used for bladder regeneration showed improved results [91-95].

Tissue engineering may generate alternatives for the current method of creating an incontinent conduit. A first attempt was made by Drewa *et al*., who created a conduit by seeding a tube from SIS with fibroblasts, and implanted this in rats [96]. This method led to unsatisfactory results, i.e. 50% of the conduits did not function due to occlusion or leakage, and only scarce ingrowth of urothelial cells was seen. Liao *et al.* seeded bladder urothelial cells on a tubularised BAM scaffold which was first implanted in omentum of rabbits and after 2 weeks used as a conduit wrapped in peritoneum [97]. This method resulted in good functional results and a confluent lining of urothelium within the construct. Basu *et al.* used smooth muscle cells derived from bladder, peripheral blood, or adipose tissue and seeded these cells on tubularised polyglycolic acid (PGA) scaffolds, coated with poly-DL-lactide-coglycolide (PLGA) [98]. This construct was wrapped in peritoneum and used as a conduit in a porcine animal model. However, functional results were not reported. Furthermore, the histological outcome was extraordinary, showing large muscle bundle formation, despite the lack of innervation in this construct, and a confluent layer of urothelium. This needs to be confirmed in a multicentre trial for external validation.

Meanwhile, a phase 1 clinical trial is started by Tengion, in which a tissue engineered conduit is used in oncologic patients who had a cystectomy, according to the concept used in the article of Basu *et al.* [99].

Tissue engineering of the abdominal wall

Tissue engineering techniques using naturally derived [100-117], synthetic degradable materials [118-120], or a combination of these (hybrid) scaffolds [121-124] have been used to close abdominal wall defects in adult animals. These studies were predominantly performed in rat models and some in rabbit or dog models. Full- or partial-thickness abdominal wall defects were surgically created, and scaffolds were used to close these defects, followed by skin closure. Commonly used naturally derived materials were decellularized tissues such as SIS [101,109,113], BAM [100,102], acellular dermal matrix (ADM) [106,107,115,116], and less often decellularized products of skeletal muscle [110,112,114], blood vessel [103], pericard [105], tunica vaginalis [111], or diaphragm [108]. The use of the reported naturally derived acellular materials generally resulted in firm connective tissue formation, degradation of the scaffold, and good integration with the native abdominal wall tissue. No infections occurred, low percentages of hernia formation were observed and a low amount of adhesions to the bowel were seen. The regenerated tissue consisted initially of inflammatory cells, followed by ingrowing fibroblasts and deposited collagen, and had good angiogenesis [100-109]. Some studies even reported ingrowth of skeletal muscle cells [101,109].

In other studies, myoblasts were harvested, cultured and seeded onto scaffolds, which were then implanted in the abdominal wall defects [110-115]. Different results were presented using this technique, i.e. some groups showed absence of muscle cells with histological evaluation [114-115], while others reported the presence of muscle cells at different time points [110-113]. Zhao *et al.* showed that seeding of bone marrow-derived mesenchymal stem cells on decellularized dermal scaffolds resulted in good tissue regeneration with muscle bundles and the absence of abdominal hernias when implanted in abdominal wall defects in rabbits. In this study, acellular scaffolds resulted in poor tissue regeneration, worse integration of the scaffold in the native tissue, and abdominal hernias [116]. Shi *et al.* loaded the growth factor FGF2 on a (skin-derived) collagen scaffold, which was implanted in an abdominal wall defect in rats [117]. This technique improved vascularization and integration of myofibers into the collagen material, and resulted in improved mechanical strength, compared to a scaffold without FGF2.

Acellular scaffolds have also been used experimentally in small numbers of neonates with large congenital abdominal wall defects inappropriate for primary closure [125-127]. SIS was used by Gabriel *et al.* combined with negative pressure wound therapy in 3 patients with complicated gastroschisis, resulting in complete epithelialization of the wound and umbilical hernias in 2 of them [125]. Van Tuil *et al.* used decellularized bovine pericard in 24 patients with gastroschisis or omphalocele, showing good integration in 97% of patients [126]. Beres *et al.* used SIS for abdominal wall repair in 13 children, which, in view of the 38% recurrence rate and 46% infection rate, was moderately successful [127].

Early detection of gastroschisis during pregnancy is possible using routine ultrasound screening and offers the opportunity for early treatment during the fetal period, to protect the bowel tissue against further secondary injury. Stephenson *et al.* successfully returned the bowel into the abdomen and repaired the abdominal wall of 2 fetal lambs with a surgically created gastroschisis using operative closure [128]. However, premature contractions resulting in premature birth is the main drawback of fetal surgery [129]. Minimal invasive treatment by laparoscopic intervention on a fetus (fetoscopy) may result in decreased complications, hereby opening the way for fetal interventions for anomalies like gastroschisis [130,131]. However, first attempts to repair a surgically created gastroschisis in a fetal lamb using fetoscopy failed [132,133]. Repair was hampered by the inflammatory fibrotic peel and distended bowel loops which enlarged the bowel volume, and resulted in severe hemodynamic compromise of the fetus when the bowel was returned into the relatively hypoplastic abdominal cavity.

Outline of this thesis

In previous studies, a large animal model for bladder exstrophy was developed, but no extensive histological analysis was performed to evaluate the comparability to the human situation [134,135]. In ***chapter 2***, we used this sheep model for bladder exstrophy and performed an extensive histological analysis of the changes in bladder wall tissue at birth.

Intrauterine treatments of patients with several congenital anomalies are developed and applied in experimental studies to decrease secondary organ damage during the fetal period, and to improve neonatal outcome. In previous work, we used fetal tissue engineering for intrauterine coverage of a surgically created neural tube defect in fetal lambs with a collagen biomatrix [136,137]. With the ongoing improvement of fetal surgery and tissue engineering techniques, fetal tissue engineering may become a new treatment option for severe congenital anomalies like bladder exstrophy. As a first step towards this approach, fetal regeneration of bladder tissue was investigated in chapter 2 as well.

In ***chapter 3***, we investigated the capability of diseased bladder to regenerate bladder tissue comparable to bladder tissue regenerated in healthy bladder. One week after birth, the bladder of lambs in which a bladder exstrophy-like lesion was prenatally created, was reconstructed using a collagen scaffold. This group was compared to a group of healthy lambs in which a scaffold was sutured in the bladder one week after birth.

In ***chapter 4***, we evaluated the effect of incorporation of growth factors VEGF165, FGF2 and HB-EGF in a collagen-heparin scaffold on bladder tissue regeneration and functionality. Scaffolds with growth factors were used to reconstruct an experimental bladder exstrophy one week after birth, and compared to the use of a scaffold without growth factors in a historical control group (described in chapter 3) and with the use of primary closure without a scaffold.
In ***chapter 5***, we describe the development of a new large diameter tubular construct and investigated its applicability and function as an incontinent urinary diversion in a preclinical porcine model.
In ***chapter 6***, we performed a prenatal repair of a full-thickness defect in the abdominal wall in fetal lambs with a surgically created gastroschisis using an acellular collagen scaffold to induce regeneration of abdominal wall tissue, and to protect the bowel.
In ***chapter 7***, the eviscerated bowel tissue of fetal lambs in which a gastroschisis was surgically created was left outside the abdominal wall and directly covered with a collagen scaffold. This strategy aimed to prevent fibrous peel and adhesion formation, and to induce cell ingrowth into the scaffold, which should ultimately result in skin or abdominal wall tissue, covering the vulnerable bowel loops.
Finally, in ***chapter 8***, a summary of this thesis and future perspectives are given involving the studies performed and developments in the field of tissue engineering for severe congenital anomalies concerning the bladder and abdominal wall.

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Chapter 2

Fetal Bladder Wall Regeneration with a Collagen Biomatrix and Histological Evaluation of Bladder Exstrophy in a Fetal Sheep Model

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Fetal Diagnosis and Therapy 2008;24:7-14

Abstract

Objectives: To evaluate histological changes in an animal model for bladder exstrophy and fetal repair of the bladder defect with a molecular-defined dual-layer collagen biomatrix to induce fetal bladder wall regeneration.

Methods: In 12 fetal lambs the abdominal wall and bladder were opened by a midline incision at 79 days' gestation. In 6 of these lambs an uncorrected bladder exstrophy was created by suturing the edges of the opened bladder to the abdominal wall (group 1). The other 6 lambs served as a repair group, where a dual-layer collagen biomatrix was sutured into the bladder wall and the abdominal wall was closed (group 2). A caesarean section was performed at 140 days' gestation, followed by macroscopic and histological examination.

Results: Group 1 showed inflammatory and maturational changes in the mucosa, submucosa and detrusor muscle of all the bladders. In group 2, bladder regeneration was observed, with urothelial coverage, ingrowth of fibroblasts and smooth muscle cells, deposition of collagen, neovascularization and nerve fibre formation. This tissue replaced the collagen biomatrix. No structural changes of the bladder were seen in group 2.

Conclusions: The animal model, as in group 1, for bladder exstrophy shows remarkable histological resemblance with the naturally occurring anomaly in humans. This model can be used to develop new methods to salvage or regenerate bladder tissue in bladder exstrophy patients. Fetal bladder wall regeneration with a collagen biomatrix is feasible in this model, resulting in renewed formation of urothelium, blood vessels, nerve fibres, ingrowth of smooth muscle cells and salvage of the native bladder.

Introduction

Bladder tissue in patients with bladder exstrophy may appear normal at birth, but a small bladder plate, fibrosis, oedema, and hypo-elasticity of the bladder tissue can be present. This can result in difficult primary closure of the bladder and impaired bladder function [1,2]. Patients with bladder exstrophy often need bladder augmentation with gastrointestinal tract tissue to enhance bladder capacity. However, this method can lead to metabolic problems, excessive mucus production, stone formation, leakage of urine, perforation and even malignancies, and sufficient bowel tissue is not always available, for example in cloacal exstrophy [3-5]. Tissue engineering techniques can be used to repair or reconstruct damaged or removed organs. During the last decade several in vitro and in vivo studies in various animal models have been performed to regenerate bladder tissue. These studies showed that new bladder tissue with the presence of urothelium, smooth muscle, vessels and nerve fibres can be formed with the use of scaffolds. Recently, the results of the first human trial showed the feasibility of tissue engineering of the human bladder, with an improvement in bladder capacity [6-9]. However, recently performed studies have made it clear that when diseased bladders were used to regenerate bladder wall tissue, worse results were seen than in normal healthy bladders, in particular far more fibrosis and less smooth muscle cells [10,11]. Therefore, there is a need for valid models to study tissue regeneration for anomalies like bladder exstrophy. In previous studies, a large animal model for bladder exstrophy was developed, but no extensive histological analysis was performed to look for changes in the bladder tissue [12-14].

Intrauterine treatment of patients with several congenital anomalies has been developed and applied in experimental studies. By this approach, further fetal organ damage can be avoided, resulting in improved neonatal outcome. In previous work, we used fetal tissue engineering for intrauterine coverage of a surgically created neural tube defect in fetal lambs with a collagen biomatrix [15,16]. With the ongoing improvement of fetal surgery and tissue engineering techniques, fetal tissue engineering may become a new treatment option for congenital anomalies like bladder exstrophy. As a first step towards this approach, fetal regeneration of bladder tissue will be evaluated in this experiment.

The aim of this study was to investigate the histological changes occurring in a large animal model for bladder exstrophy and to evaluate the possibility and extent of fetal bladder tissue regeneration in this animal model. For this purpose, a sheep model for bladder exstrophy was used and extensive histological analyses of the changes in bladder wall tissue were performed at birth. In the second group, the bladder defect was repaired immediately after creation with a molecularly defined biocompatible and biodegradable dual-layer collagen biomatrix, and regeneration of the fetal bladder wall was evaluated.

Material and Methods

Preparation of Collagen Biomatrices

The molecularly defined, biocompatible and biodegradable dual-layer collagen biomatrices were made from insoluble type I collagen purified from bovine Achilles tendon [17]. The biomatrix consisted of a porous layer and a dense film layer. A 0.8% (w/v) type I collagen suspension was shaken overnight in 0.25 M acetic acid at 4°C and homogenized on ice using a Potter-Elvehjem homogenizer. Air bubbles were removed by centrifugation at 250*g* for 10 min at 4°C. The suspension was slowly poured into a plastic mould (4 ml per Ø 32 mm), and air-dried for 3 days at 22°C to prepare a flat film layer. These films were incubated for 60 min in 4 ml 0.25 M acetic acid. The acetic acid was then removed, and a suspension of 4 ml 0.8% type I collagen in 0.25 M acetic acid was poured on top of the films, quickly frozen at -80°C and lyophilized in a Zirbus lyophilizer (Bad Grund, Germany). Scaffolds were cross-linked using 33 mM 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide and 6 mM N-hydroxysuccinimide in 50 mM 2-morpholinoethane sulphonic acid pH 5.0 containing 40% ethanol (5 ml per Ø 32 mm) for 4 h at 22°C. Scaffolds were then washed with 0.1 M Na_2HPO_4 , 1 M NaCl, 2 M NaCl and MilliQ water, frozen in ethanol/CO_2 again and lyophilized [18,19]. Biomatrix morphology was assessed by scanning electron microscopy (Figure 1) [18,19]. The mean average pore size of the top side of the porous layer was 106 ± 22 µm and of the cross-section 123 ± 34 µm (average of 100 pores of 3 individually prepared biomatrices). Cross-linking was verified by its amine group content, and 48% of the amine groups were used in the cross-linking process [18]. Before implantation the matrices were washed in 70% (v/v) ethanol and sterile phosphate-buffered saline.

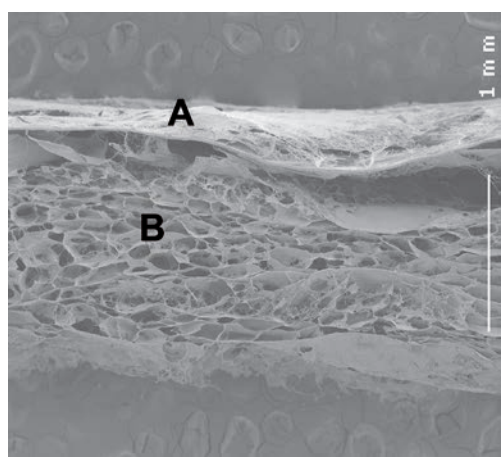

Figure 1. *Dual-layer collagen biomatrix. Scanning electron microscopy. A = Film layer; B = porous layer.*

Surgical Procedures

After obtaining approval from the Ethical Committee on Animal Research, 12 pregnant sheep (Dutch Texels breed) were operated at 79 days' gestation (full term 140-147 days). Anaesthesia was induced by intravenous injection of 30 mg/kg pentobarbital and 1 ml atropine (0.5 mg/ml) and, following endotracheal intubation, maintained with 2% isoflurane and O_2/air ventilation at a respiration rate of 16 per minute. The uterus was exteriorized through a midline abdominal incision. A hysterotomy was performed and the lower part of the fetal body was exposed. In case of twin or triplet pregnancy only 1 fetus was operated to avoid additional risk of complications. In female fetuses, a vertical infraumbilical median incision was made through skin and fascia of the abdominal wall and in male fetuses, a paramedian incision was made next to the urethra. The bladder was exposed and an incision of approximately 1.5 cm was made into the anterior bladder wall.

In the first group of 6 fetuses (5 male, 1 female), a bladder exstrophy was surgically created. The edges of the bladder wall were sutured to the abdominal wall with 6-0 polyglecaprone (Monocryl® , Ethicon Inc., Sommerville, N.J., USA) interrupted sutures, resulting in an exstrophied bladder measuring approximately 1.5 x1 cm (Figure 2A).

In the second group of 6 fetuses (3 male, 3 female), the dual-layer collagen biomatrix, measuring 1.5 x 1 cm, was placed into the bladder defect (film layer at the luminal site) and sutured to the bladder wall with 7-0 polyglactin (Vicryl®, Ethicon Inc.) running sutures (Figure 2B). Four 6-0 polypropylene (Prolene®, Ethicon Inc.) non-resorbable marking sutures were placed at the edges of the bladder. The fetal abdominal wall was closed with 6-0 polyglecaprone interrupted sutures after replacing the bladder into the abdominal cavity.

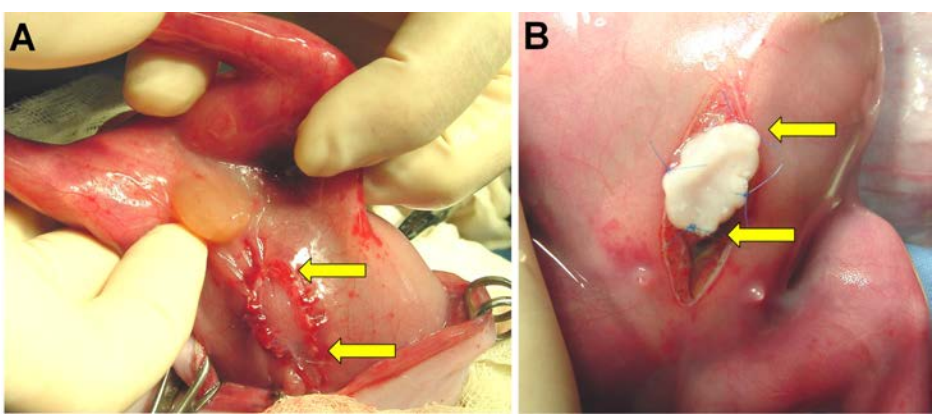

Figure 2. *Surgically created fetal bladder exstrophy defect.* ***A.*** *Uncovered defect (bladder between arrows).* ***B.*** *Biomatrix (between arrows) sutured in exstrophied bladder, before closure of abdominal wall.*

After the surgical procedure the fetus was returned into the uterus and amniotic fluid volume was restored with warm sterile saline together with amoxicillin 250 mg. The uterus was closed in 2 layers with a 2-0 polyglactin running suture. Sodium-penicillin (1,000,000 IU) was instilled into the intra-abdominal space and the abdominal wall was closed in 2 layers using 1 polyglactin interrupted sutures. Depomycine (20 mg/kg s.c.) was initiated preoperatively and maintained postoperatively for 3 days.
At 140 days' gestation, 61 days after surgery, the lambs were delivered by caesarean section under local anaesthesia with 20-30 ml lidocaine 2%, administered subcutaneously and intramuscularly.

Neonatal Outcome and Evaluation
After birth the lambs were examined macroscopically and photographed, and sacrificed with medetomidine (0.5 mg intramuscular) and pentobarbital (60 mg/kg intracardial). In group 2, a cystogram was performed after sacrifice. Subsequently, the bladders were removed and the outside and inside of the bladders were inspected. The kidneys were examined for signs of infection, hydronephrosis, and stone formation.

Histological Staining
In all lambs tissue samples of the posterior bladder wall were taken. Tissue samples of normal bladders from 5 lambs that had undergone a fetal operation in another study served as control group. In group 2, the tissue between the marking sutures at the site of the implanted biomatrix was also taken out. Renal tissue of all lambs was processed. Tissue samples were fixated in 10% buffered formalin and paraffin-embedded for routine histological processing. Sections (4 µm) were cut and stained with haematoxylin and eosin and Masson's trichrome. Tissues from the posterior wall of all lambs were examined for changes in the urothelial, submucosal and detrusor muscle layers. Tissue from the site of biomatrix implantation was examined for epithelialization, smooth muscle cell growth and neovascularization within the biomatrix. Quantitative morphometric analysis of collagen- to smooth muscle content was performed by applying a point-counting technique on 5 representative colour pictures of the samples from the posterior bladder wall of all 3 groups, stained by Masson's trichrome. Immunohistochemical stainings were performed using α-smooth muscle actin and desmin for staining muscle cells (only group 2) and S-100 staining to visualize nerve fibres. Renal tissue was examined for inflammatory changes or dilated nephrons.

Statistical Analysis
Statistical analysis of the tissue quantification was performed with SPSS 12.0 for Windows (SPSS, Chicago, Ill., USA), using a one-way ANOVA. $p<0.05$ was considered statistically significant.

Results

Eleven of the 12 operated fetuses (92%) were born alive. One fetal demise occurred in group 1 in which an evisceration, next to the exstrophied bladder, with bowel torsion was seen. No maternal deaths occurred. In the 5 lambs of group 1, the exstrophied bladder was visible in the abdominal wall defect after birth, measuring approximately 4 x 3 cm (Figure 3). The bladder tissue macroscopically appeared inflamed, with ulceration and erythema of the bladder mucosa; a small polyp was visible in 1 lamb.

Histological examination of the bladder wall showed distinct changes of the mucosa in 4 fetuses, the submucosal tissue in all fetuses, and the detrusor muscle in 4 fetuses (Table 1). In 3 of 5 lambs the bladder tissue showed ulceration of the urothelial layer, with granulation tissue and chronic reactive inflammation. In 1 lamb squamous metaplasia was present. Submucosal fibrosis was apparent and the number of capillaries was increased. The detrusor muscle showed atrophy and fibrosis in the inner layer (Figure 4A,B). S-100 staining did not differentiate well enough between nerve fibres and smooth muscle cells to quantify the nerve fibre content, as was previously performed in human bladder exstrophy [20].

Figure 3. *Bladder exstrophy after birth.*

Table 1. *Histological results of the bladder exstrophy group after birth*

Lamb	Mucosa	Submucosa	Detrusor muscle
1	Normal	Capillaries ++ Scarce fibrosis	Atrophy of inner layer Fibrosis
2	Local erosion Chronic reactive inflammation and granulation tissue formation	Capillaries + Fibrosis	Normal
3	Massive erosion Chronic reactive inflammation and granulation tissue formation	Capillaries ++ Large vessels + Fibrosis	Atrophy of inner layer Fibrosis
4	Local erosion Chronic reactive inflammation and granulation tissue formation	Capillaries ++ Fibrosis Scarce chronic inflammation	Atrophy of inner layer Fibrosis
5	Squamous metaplasia	Capillaries ++ Fibrosis	Atrophy of inner layer Fibrosis

[+]= increase in amount; [++]= large increase in amount, compared to normal bladder

In all lambs of group 2 the abdominal wall was closed, only a small scar was visible in the skin. The cystograms showed bladders with a normal shape; no diverticulum or leakage was seen (Figure 5A). In 2 lambs the bladder was adhered to the abdominal wall, in the other 4 lambs no adhesions existed and the area of newly formed bladder tissue with new vessel formation was clearly visible due to the marking sutures, with an average diameter of 1.0 cm (Figure 5B,C). Stone formation was absent in all lambs. Bladder tissue had replaced the porous layer of the biomatrix; however, the bladder wall was thinner than the native bladder wall, with an average of 24 mm in this regenerated tissue compared to an average of 29 mm in native bladder tissue. The film layer of the biomatrix was not degraded and was still present on the inside of the bladder wall.

Histological examination of the regenerated tissue showed multi-layered urothelium lining the entire region of the regenerated part of the bladder, which was indistinguishable from native urothelium (Figure 6A,B). Capillaries and well-formed blood vessels were visible throughout the entire thickness of the regenerated tissue (Figure 6A-C). Fibroblasts, myofibroblasts and collagen deposition were abundant in the submucosal layer. The porous layer of the collagen biomatrix had been degraded; only small remnants remained, encapsulated by histiocytic and fibroblastic cells (Figure 6C). Smooth muscle cells were apparent, with random arrangement in fascicles instead of the well organized muscle layer of the native bladder, and could be differentiated from myofibroblasts by morphologic appearance (Figure 6D).

Alpha-smooth muscle actin did not differentiate between smooth muscle cells and myofibroblasts, and almost the whole submucosa of the regenerated tissue was positive for this staining. Ingrowth of nerve fibres was identified (Figure 6E). The posterior wall, representing the native bladder, did not show any histological changes. The kidneys of both groups did not show any macroscopic or histological abnormalities.

For quantitative morphometric analysis, we assumed the areas occupied by collagen and smooth muscle to be 100% on each specimen. Analysis revealed a significant increase in collagen in exstrophic bladders (group 1) with a collagen-to-smooth muscle ratio of 1.7 (62% collagen to 38% smooth muscle), compared to a ratio of 0.7 (41% to 59%) in the control group ($p < 0.001$), and 0.9 (46% to 54%) in the bladders with a biomatrix (group 2) ($p < 0.001$). No statistically significant difference existed between group 2 and the control group.

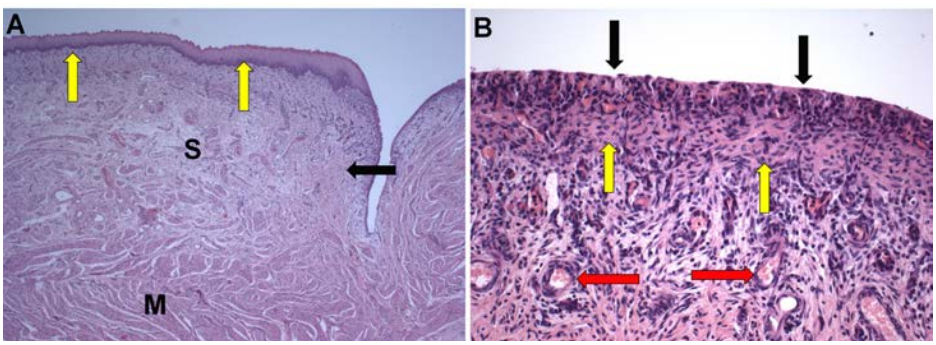

Figure 4. *Histological pictures of the bladder exstrophy after birth.* ***A.*** *Squamous metaplasia of the urothelial layer (yellow arrows), increased number of capillaries and fibrosis (black arrow) in the submucosal layer (S); detrusor muscle (M) with atrophy and fibrosis, most obvious in the inner layer of the muscle. HE. Original magnification x25.* ***B.*** *Mucosa and submucosa, showing ulceration of the urothelium (black arrows), fibrosis (yellow arrows) and increased number of capillaries (red arrows). HE. x200.*

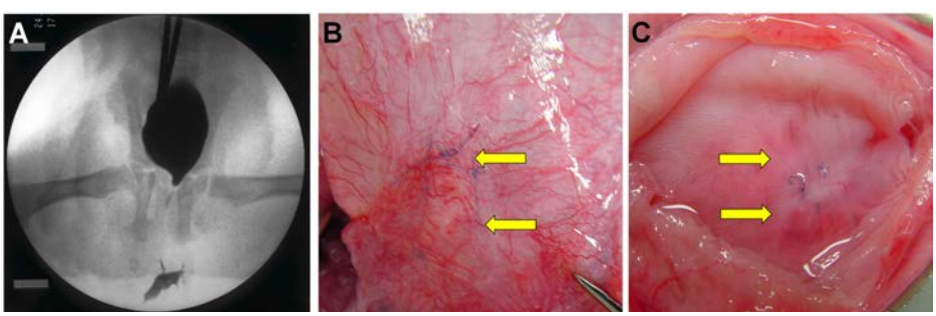

Figure 5. *Fetal bladder with biomatrix after birth.* ***A.*** *Cystogram.* ***B****. Outer side of the bladder after infusion of PBS. Regenerated tissues can be seen between arrows.* ***C.*** *Inner side of the bladder. Regenerated tissues can be seen between arrows.*

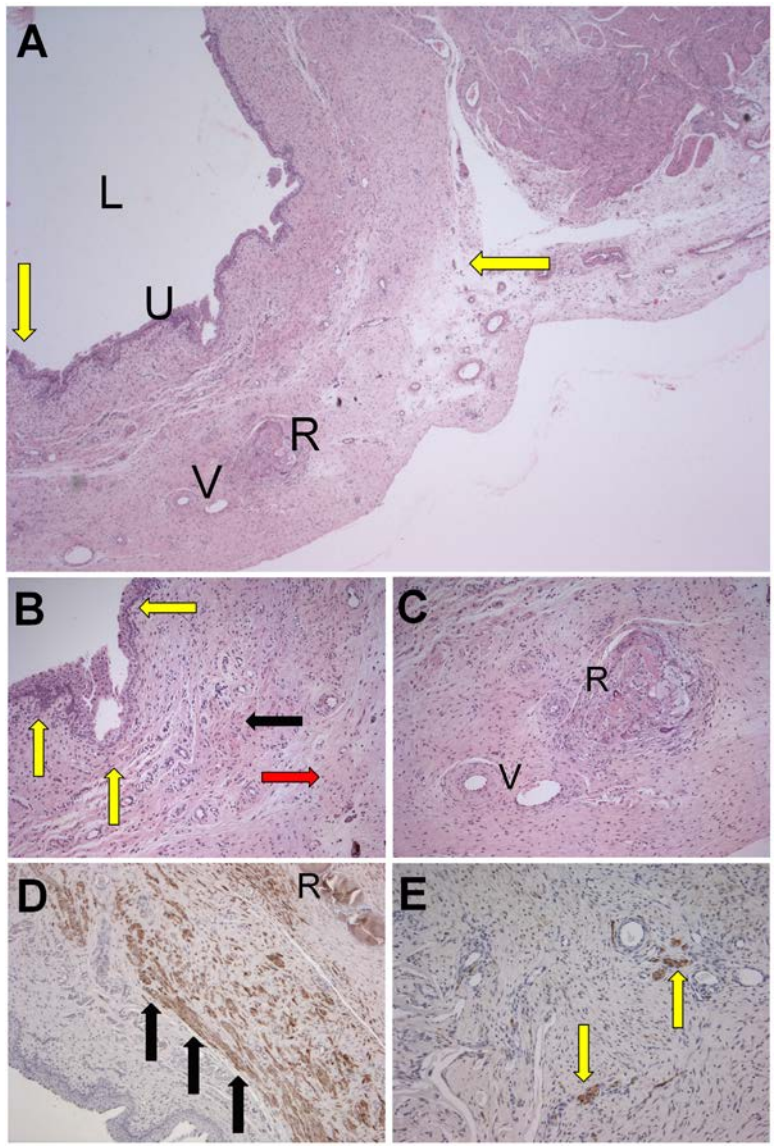

Figure 6. *Histological pictures of the regenerated tissues from the bladder with biomatrix.* ***A.*** *Overview of the regenerated tissue (between arrows). L = Bladder lumen; U = urothelium; R = remnant of the biomatrix; V = vessels. HE. Original magnification x25.* ***B.*** *Urothelial layer (yellow arrows), smooth muscle cells (black arrow) and fibroblasts with collagen (red arrows). HE. x100.* ***C.*** *Well-developed blood vessels (V), capillaries and a remnant of the collagen biomatrix (R). HE. x100.* ***D.*** *Smooth muscle cells (black arrows), myofibroblasts (yellow arrows) and urothelium (U) in the regenerated tissue, remnant of biomatrix (R). Desmin. x100.* ***E.*** *Nerve fibers (arrows) in the submucosa. S-100. x100.*

Discussion

In fetuses with bladder exstrophy, bladder tissue is exposed to the amniotic fluid, containing possibly harmful substances, and the bladder is defunctionalized. Prolonged exposure to the amniotic fluid and lack of bladder cycling cause inflammatory and maturational changes to the bladder wall, respectively [20-22]. At birth the bladder can be small in size, with fibrosis, oedema and polyps [1,2]. Tissue samples of neonates and older patients reveal severe histological abnormalities, even after primary or delayed closure of the bladder [23-25]. Acute or chronic inflammation can be found, with ulceration and squamous metaplasia, cystitis cystica and cystitis glandularis. In the submucosa, fibrosis and dilated vessels are present. In the detrusor muscle, fibrosis and disorganization of the muscle arrangement may occur. An increase in the collagen-to-smooth muscle ratio in exstrophic bladders has been described [21,22].

In this study we developed a fetal animal model to imitate the bladder changes in patients with bladder exstrophy, and evaluated the histological alterations in the different layers of the bladder. We opened the bladder and sutured it into the abdominal wall, as was previously described by Fauza *et al.* [12]. In contrast to Slaughenhoupt *et al.* [13,14], we neither performed an osteotomy of the symphysis, nor opened the urethra of the lambs, because we were interested only in changes occurring in the bladder wall. Our survival rate of 92% was high and the model was highly reproducible. The histological results showed remarkable resemblance with the above-described changes in bladder tissue of human congenital bladder exstrophy. Therefore, this animal model can be very useful for further studies of fetal bladder development in bladder exstrophy, as well as for evaluation of new or improved treatment options to salvage or regenerate bladder tissues in this anomaly. Moreover, recent animal studies underline that diseased bladders do not seem to have the same regenerative capacities as normal bladders, which emphasizes the need for models with diseased bladders for reliable evaluation of bladder regeneration [10,11].

Due to the alterations in bladder tissue, primary closure can be difficult in patients with bladder exstrophy and bladder function is often impaired [1,2]. Some patients need bladder augmentation, and tissue engineering can be a promising alternative for the use of gastrointestinal tissue. Several *in vitro* and *in vivo* studies in various animal models have shown the postnatal regeneration of the essential tissues in the bladder wall [6-8]. Recently, the first human clinical trial was conducted, showing the feasibility of the concept in human bladders and improvement of bladder capacity [9]. A method for fetal tissue engineering was developed by Fauza *et al.* [12], who created an experimental congenital anomaly in the fetal lamb and harvested cells from the target organ by a biopsy. These cells were expanded in vitro and seeded onto a scaffold, for subsequent use in neonatal surgery to regenerate tissue in the animal. In previous work we successfully used a collagen scaffold to cover a surgically

created neural tube defect in fetal lambs [15,16]. Fetal closure of the bladder could result in less damage to the bladder at birth, due to protection against the amniotic fluid and assumed induction of bladder cycling. By using tissue-engineering techniques in fetal tissues, one could take advantage of the large regenerative capacities and the fast cell growth of fetal tissues [12,26]. Another advantage is the smaller amount of tissue that has to be regenerated because of the smaller size of the fetal bladder, and adequate neovascularization is then more likely to occur throughout the entire biomatrix.

In this experiment we studied the effect of fetal bladder tissue regeneration by the immediate repair of a bladder defect with a collagen biomatrix, as a first step towards intrauterine coverage or repair of bladder exstrophy. No sham-operated control group was added, because we felt this would not have provided extra information about the regeneration of tissue. Urodynamic studies were not performed because the main interest in this study was to evaluate the feasibility of regenerating bladder tissue, and the technical inability to catheterize male lambs due to an s-curvature in the urethra.

We used a molecularly defined, biocompatible and biodegradable dual-layer collagen biomatrix. Collagen scaffolds have the advantage of good cell-binding capacities, low antigenicity and good biodegradability [17]. Biodegradability is an especially important issue in tissue engineering for children; degradable products will be replaced by new tissue, which will grow with the child. This biomatrix is a modification of the biomatrix previously used for bladder wall regeneration in an adult rabbit model [7]. The modification consisted of adding a thin layer of collagen with less porosity and higher tensile strength to the porous layer, to increase the total tensile strength of the biomatrix. Furthermore, creating a barrier between the regenerating tissue and the cell-toxic urine with the impermeable film layer might have improved the regeneration process in the porous layer of the biomatrix [10].

Histological examination of the regenerated bladder wall revealed regeneration of urothelium, neovascularization, smooth muscle formation and nerve fibres. Although the newly formed tissues did not perfectly resemble the constitution and organization of native tissues at birth, one might argue that further anatomical improvement during the postnatal period would have occurred if the neonates had not been sacrificed. The porous layer of the collagen biomatrix had largely been degraded, but the film layer was still intact. Due to its lower porosity the time for degradation is prolonged and ingrowth of cells did not occur. Moderate graft shrinkage was reported in earlier studies and also occurred in our study. This is caused by the activity of (myo-)fibroblasts which enter the biomatrix.

This study showed that fetal tissue engineering of the bladder, with the use of a collagen biomatrix, was feasible in an animal model and bladder wall regeneration occurred. Immediate repair of the bladder defect during the fetal period salvaged native bladder tissue in this model. With fetal repair further damage and probably improvement of the bladder tissue in the fetus with bladder exstrophy can

be accomplished. The major drawback of fetal surgery at present is the risk of complications leading to premature delivery [27,28]. However, one might speculate that if fetal surgical techniques and techniques of tissue engineering improve further in the future, the fetus with a congenital anomaly like bladder exstrophy might benefit from fetal tissue engineering to regenerate damaged or absent tissues.

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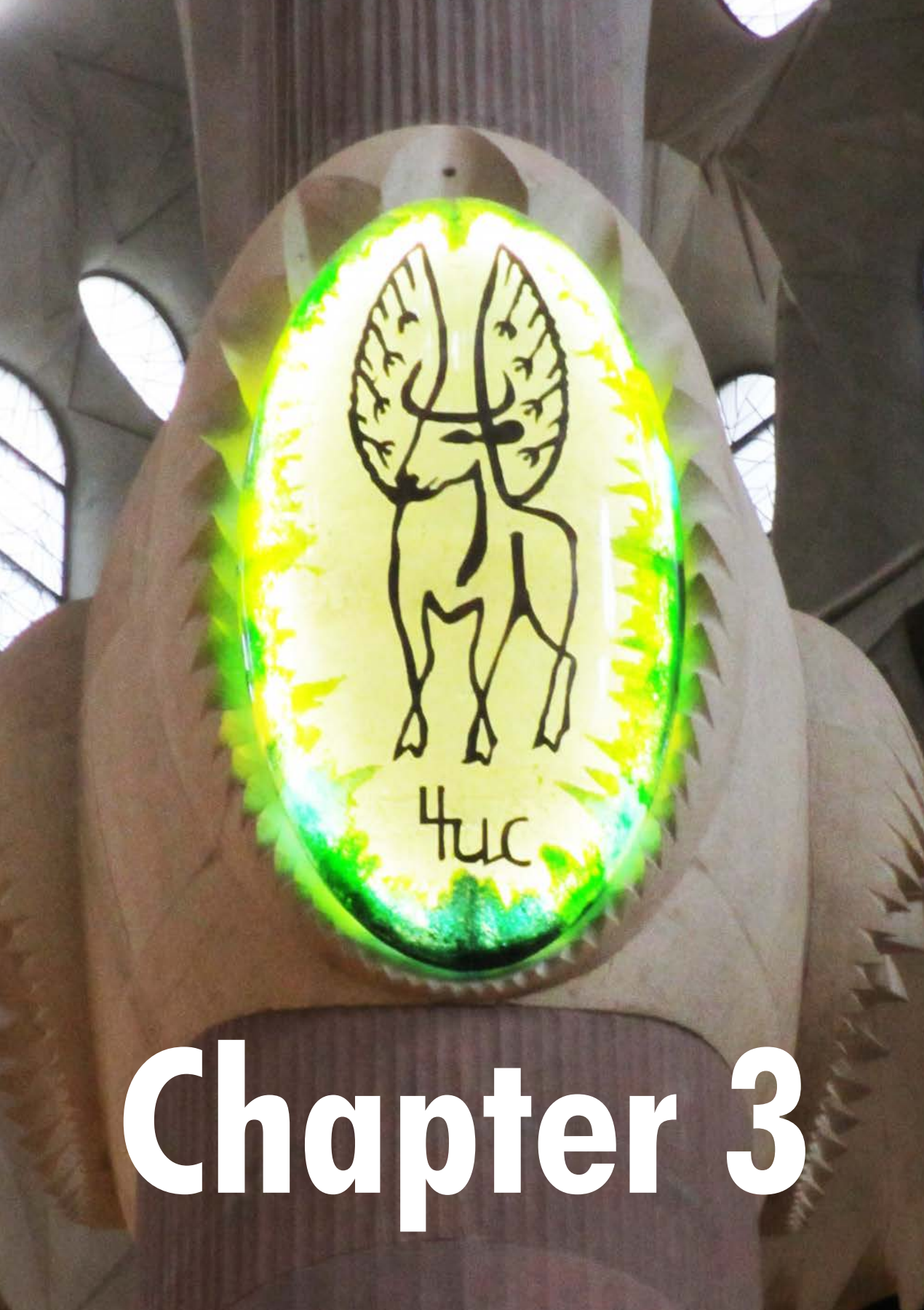

Chapter 3

Tissue Engineering of Diseased Bladder using a Collagen Scaffold in a Bladder Exstrophy Model

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British Journal of Urology International 2014

Abstract

Objectives: To compare the regenerative capacity of diseased bladder in a large animal model for bladder exstrophy to regeneration in healthy bladder using a highly porous collagen scaffold.

Materials and Methods: Highly porous bovine type I collagen scaffolds with a diameter of 32 mm were prepared. In 12 fetal sheep a bladder exstrophy was surgically created at 79 days' gestation. Lambs were born at full term (140 days' gestation). After 1 week the bladder lesion was reconstructed and augmented with a collagen scaffold (group 1). In 9 normal newborn lambs the bladder was augmented with a collagen scaffold 1 week after birth (group 2). Functional (video urodynamics) and histological evaluation was performed 1 and 6 months after surgery.

Results: Survival rate was 58% in group 1 and 100% in group 2. Cystograms were normal in all animals, besides low grade reflux in both groups. Urodynamics showed comparable capacity between both groups and a trend to lower compliance in group 1. Histological evaluation at 1 month revealed a non-confluent urothelial layer, an immature submucosa, and initial ingrowth of smooth muscle cells. At 6 months both groups showed normal urothelial lining, standard extracellular matrix development, and smooth muscle cell ingrowth.

Conclusion: Bladder tissue regeneration with a collagen scaffold in a diseased bladder model and in healthy bladder resulted in comparable functional and histological outcome, with good quality of regenerated tissue involving all tissue layers. Improvements may still be needed for larger augmentations or more severely diseased bladders.

Introduction

Children with congenital anomalies like bladder exstrophy or myelomeningocele can develop low-capacity and high-pressure bladders, often requiring bladder augmentation [1]. Bladder augmentation is most often performed with gastrointestinal tissue. However, this method can lead to metabolic disturbances, infections, excessive mucus production, stone formation, perforation and even malignancies, and sufficient bowel tissue is not always available [1-4].

Tissue engineering techniques aim to repair or replace damaged or absent organs by inducing regeneration processes, and may be an alternative for bladder augmentation. *In vitro* and *in vivo* studies in various animal models have been performed to regenerate bladder tissue. These studies showed that new bladder tissue, including urothelium, smooth muscle, vessels and nerve fibres, can be generated with the use of tissue engineered constructs [5-7].

In the first clinical trial related to bladder tissue engineering, performed by Atala *et al.,* neuropathic bladders of 7 children with myelomeningocele were augmented [8]. Particularly the composite scaffold of collagen and polyglycolic acid seeded with cultured autologous urothelial and smooth muscle cells and wrapped in omentum, to support vascularity, showed promising results. Subsequently, multi-centre phase II clinical studies were performed in pediatric patients with a neuropathic bladder due to spina bifida and in adult patients with spinal cord injury [6,7,9], showing the feasibility of the technique. However, refinement of the techniques are needed to further improve capacity and compliance before it can replace the current methods of bladder augmentation [6-10].

Preclinical animal studies have generally been performed in healthy bladders. However, translation to patients is hampered by observations that physiological function of smooth muscle cells of neuropathic bladders appear altered [11], and cultured urothelial and smooth muscle cells from patients with a neuropathic bladder or bladder exstrophy behave dissimilar from normal cells [12-20]. This may have implications when used for tissue engineering purposes. Indeed, two studies performed in an animal model for diseased bladder demonstrated that tissue engineering of diseased bladder led to much more fibrosis, less smooth muscle cells and poor functionality [21,22]. Therefore, the aim of this study was to investigate the capability of diseased bladder to generate bladder tissue comparable to bladder tissue generated in healthy bladder. We used a large animal model for bladder exstrophy, developed in fetal lambs [23,24], which has extensive similarity with human bladder exstrophy, as judged in previous work by morphological and histological analysis directly after birth [25]. A highly porous, biocompatible and biodegradable scaffold of bovine type I collagen was used for implantation [26-28].

Materials and Methods

This study was approved by the Ethical Committee on Animal Research of the Radboud university medical center, Nijmegen, the Netherlands (RU-DEC 2007-113).

Collagen scaffolds

Preparation of collagen scaffolds

Round collagen scaffolds [29] with a diameter of 32 mm were prepared from insoluble bovine type I collagen fibrils as previously described [25]. Scaffolds were disinfected in 70% (v/v) ethanol and washed with sterile phosphate buffered saline (PBS).

Biochemical characterization of collagen scaffolds

The ultrastructure of the scaffolds was visualized by scanning electron microscopy (SEM) using a JEOL JSM-6310 SEM apparatus operating at 15 kV. The degree of crosslinking was determined spectrophotometrically from the amine group content using 2,4,6-trinitrobenzene sulfonic acid [30,31].

Surgical procedures

Prenatal operations

Group 1 (bladder exstrophy lambs): 12 pregnant sheep (Dutch Texel breed) were operated at 79 days' gestation (full term 140-147 days). Animals were pre-medicated with intramuscular (IM) injection of midazolam (0.5 mg/kg, Roche, Woerden, the Netherlands). General anaesthesia was induced by intravenous (IV) injection of propofol (5 mg/kg, B. Braun, Melsungen, Germany), followed by tracheal intubation, and maintained with 1.5% isoflurane (Nicholas Piramal, London, UK). For analgesia, flunixin (2 mg/kg, Intervet, Boxmeer, the Netherlands) and sufentanil (4 µg/kg, Janssen Cilag BV, Tilburg, the Netherlands) were given IV, followed by a maintenance dosage sufentanil of 2 µg/kg/h. Fetal anesthesia was achieved by transplacental passage of the medications administered to the ewe. The fetal operation was earlier described in Roelofs *et al.* [25]. In short: a laparotomy and hysterotomy were performed on the ewe. A laparotomy was performed on the fetus and the bladder was exposed. An incision of approximately 1.5 cm was made into the anterior bladder wall. The symphysis pubis, bladder neck and urethra were left intact. The edges of the bladder wall were sutured to the abdominal wall with 6-0 poliglecaprone (Monocryl®, Ethicon Inc.; Sommerville, NJ, USA) interrupted sutures, resulting in an exstrophied bladder measuring approximately 1.5 x 1 cm (Figure 1A). Subsequently the fetus was returned into the uterus and the uterus and abdominal wall were closed. Buprenorphine (10 µg/kg, IV, Schering Plough, Segre, France) was given once, and flunixin (2 mg/kg, IM) for three days as postoperative analgesia. Parturition was induced at 140 days' gestation with Dexadreson® (12-15

ml, IM, Intervet, Boxmeer, the Netherlands) and Trilostane (60 mg, oral, Janssen-Cilag GmbH, Tilburg, the Netherlands), resulting in vaginal delivery.

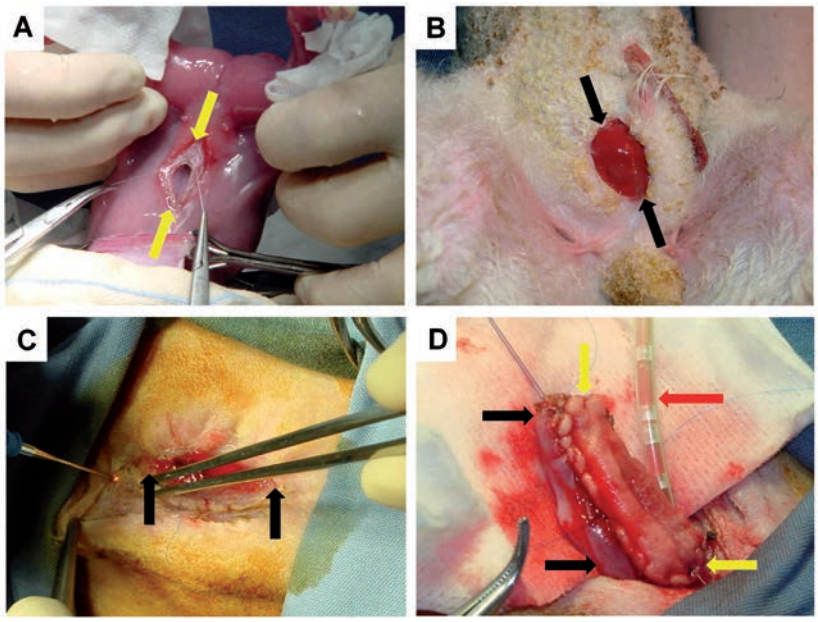

Figure 1. *Surgical procedures:* ***A.*** *Prenatal operation: fetal bladder was opened (between arrows) and sutured to the abdominal wall.* ***B.*** *Postnatal macroscopic result, bladder plate visible between arrows.* ***C.*** *Postnatal operation: surgical detachment of bladder plate (between arrows) from abdominal wall.* ***D.*** *Collagen scaffold (between yellow arrows) sutured to bladder plate (between black arrows). Suprapubic catheter (red arrow).*

Postnatal operations

The 7 surviving lambs of group 1 were operated one week after birth (Figure 1B). Nine normal newborn lambs were operated one week after birth forming the control group (group 2). Anaesthesia was performed as described above. In group 1 the bladder plate was surgically detached from the abdominal wall, and 3-4 mm of the transition zone between the bladder plate and the abdominal wall was removed (Figure 1C). In group 2 a laparotomy was performed, the bladder was identified and the anterior wall was opened. Subsequently, in both groups the round collagen scaffold with a diameter of 32 mm was sutured onto the bladder plate using 6-0 poliglecaprone running sutures (Figure 1D). Four 6-0 polypropylene (Prolene®, Ethicon Inc.) nonresorbable marking sutures were placed at the edges of the scaffold. A 5Fr suprapubic catheter (Cystofix®, B. Braun) was placed and fixed to the bladder using 6-0 poliglecaprone. The omentum was sutured to the bladder, to cover the collagen scaffold, using 6-0 poliglecaprone. The abdominal wall was closed using 2-0 polyglactin (Vicryl®, Ethicon Inc.) interrupted sutures. The suprapubic

catheter was fixed to the skin using 2-0 polyglactin sutures, and was removed after 3 weeks. Flunixin (2mg/kg, IM) was given during 3 days as postoperative analgesia. Enrofloxacin (5%, 0.5 ml/10 kg, subcutaneous, Bayer B.V., Mijdrecht, the Netherlands) was given as antibiotic once daily during 7 days.

Neonatal outcome and evaluation

The animals were at random evaluated after 1 or 6 months to study the regenerative process in time. Anesthesia was performed as described above. Video urodynamic evaluation was performed using the MMS Solar system (MMS, Enschede, the Netherlands) and a Philips BV-25 C-arm and image identifier (Philips, Eindhoven, the Netherlands). A double lumen 6Fr catheter (Medtronic, Heerlen, the Netherlands) was placed in the bladder, through the urethra in female and through an incision in the urethra in male lambs. A double lumen 9Fr catheter (Bel Medical B.V., Zwolle, the Netherlands) was placed in the rectum. The bladders were slowly filled with iodinated contrast fluid (Xenetix® 300 (Guerbet Nederland BV., Gorinchem, the Netherlands) diluted with PBS 1:1). The abdominal leak point pressure was measured in female lambs and the point of steep increase of intravesical pressure in male lambs, as well as bladder capacity at this point. Compliance was calculated from these measurements. One to 3 representative evaluations were performed in each lamb. After evaluation the lambs were sacrificed with medetomidine (0.5 mg IM, Orion pharma, Espoo, Finland) and pentobarbital (60 mg/kg intracardial, AST Pharma, Oudewater, the Netherlands). The bladder was removed and the outside and inside of the bladder were inspected. Kidneys were examined for signs of infection, hydronephrosis, or stone formation.

Histological staining

In both groups tissue samples were obtained of the native posterior bladder wall and of the regenerated bladder wall, fixed in 4% (v/v) buffered formalin and paraffin-embedded. Sections (4 µm) were cut with a microtome and stained with hematoxylin & eosin (H&E) and Masson's trichrome staining. For immunohistochemistry sections were deparaffinized. Endogenous peroxidase was blocked with 3% (v/v) H_2O_2/PBS. Antigen retrieval methods are shown in table 1. Slides were pre-incubated with 5% goat serum and incubated with the antibody of interest (Table 1). Slides were incubated with poly-HRP-Anti Ms IgG (Immunologic, Duiven, the Netherlands), color development was performed using power DAB (3,3-diaminobenzidine) (Immunologic), and counterstained with Mayer's hematoxylin (Fluka Chemie, Buchs, Switzerland).

Renal tissue of all lambs was processed and examined for inflammatory changes or dilated nephrons by H&E. Sections were analysed using an Zeiss Axioskop FS light microscope.

Table 1. *Antibodies used for immunohistological evaluation*

Antigen	Antibody	Source	Dilution	Antigen retrieval
CK 7	RCK105	MUbio BV	1:10	A
CK 10	RKSE-60	MUbio BV	1:5	A
CK 14	LL002	Thermo Fisher scientific	1:100	A
CK 20	CK20	Immunologic	1:200	A
Pancytokeratin AE1/AE3	AE1/AE3	Thermo Fisher scientific	1:800	B
Ki-67	SP6	Thermo Fisher scientific	1:200	A
Vimentin	V-9	BioGenex	1:2000	C
α-SMA	1A4	Sigma-Aldrich	1:15000	C
Desmin	[33]	BioGenex	1:200	C
Smoothelin	R4A	Santa Cruz Biotechnology	1:150	A

A: Heat mediated in sodium citrate buffer(10mM; pH6.0; 10min); B: with 0.1% pronase (30 min at RT); C: without antigen retrieval

Statistical analysis

Data analysis of the urodynamic results was performed with SPSS 18.0 for Windows, and expressed as mean ± standard deviation. Statistical analysis was performed using the independent samples t-test for equality of means. $P < 0.05$ was considered statistically significant.

Results

Scaffolds

Porous collagen scaffolds with rounded pores were obtained [29]. Two thirds of the primary amine groups remained after crosslinking compared to non-crosslinked scaffolds, indicating that one third of amine groups had been chemically crosslinked. Further descriptions are available in previous work [29].

Animal surgery

The survival rate was 58%, 7 out of 12 operated fetuses of group 1 were born alive. Three fetuses had an intra-uterine demise, for which no reason was found, and 2 fetuses died during delivery. No maternal deaths occurred. These 7 lambs were operated and survived until the end of the study (survival rate 100%). Three lambs were evaluated 1 month after the postnatal operation, 4 lambs were evaluated after 6 months. All 9 lambs in group 2 survived until the end of the study (survival rate 100%). Four were evaluated 1 month after the operation, 5 were evaluated after 6 months.

Functional evaluation

After removal of the catheter all lambs voided normally, no incontinence or signs of urinary tract infection were seen.

Urodynamic evaluation did not show detrusor overactivity in any lamb. Results are shown in Table 2. The increase in capacity between 1 and 6 months was statistically significant in group 1 ($p = 0.02$), but not for group 2 ($p = 0.10$). No difference in

Table 2. *Results of urodynamics performed in lambs*

Group 1 (Bladder exstrophy)			Group 2 (Controls)		
1 month					
Lamb	Capacity (ml)	Compliance (ml/cmH_2O)	Lamb	Capacity (ml)	Compliance (ml/cmH_2O)
1	50	2.6	1	21	1.0
2	14	0.5	2	66	6.8
3	NA	NA	3	44	2.0
			4	55	2.6
Mean	32	1.5		46	3.1
SD	25	1.4		19	2.6
6 months					
Lamb	Capacity (ml)	Compliance (ml/cmH_2O)	Lamb	Capacity (ml)	Compliance (ml/cmH_2O)
1	82	2.1	1	91	3.9
2	73	2.2	2	116	7.4
3	70	2.1	3	88	2.7
4	94	2.8	4	38	3.4
			5	NA	NA
Mean	80	2.3		83	4.4
SD	11	0.3		33	2.1

NA = not available (Suprapubic catheter was placed, leakage occurred during urodynamics); SD = standard deviation

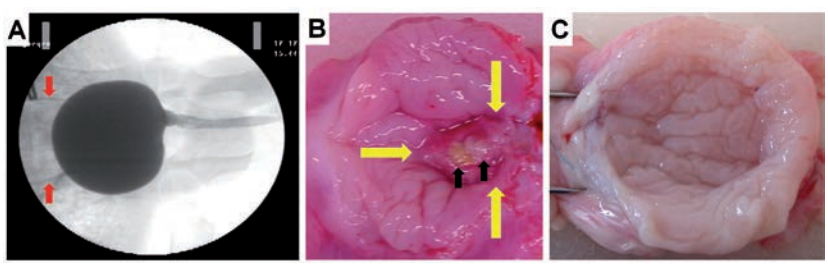

Figure 2. A. *Cystogram showing no abnormalities except bilateral reflux (red arrows),* ***B.*** *Macroscopic view inside of bladder at 1 month. Regenerated tissue between yellow arrows. Remnant of scaffold visible at black arrows.* ***C.*** *Macroscopic view inside of bladder at 6 months. No difference between regenerated and native bladder.*

capacity was seen between both groups at both time points. Comparing the compliance between the 2 groups at the different time points showed a decrease in compliance in group 1, but this was not statistically significant at both time points (p= 0.41 at 1 month; p = 0.10 at 6 months).
Cystograms made during the filling phase showed no structural abnormalities of the bladder, e.g. no diverticulum or leakage (Figure 2A). In almost all lambs in both groups low grade reflux was seen at higher intravesical pressures.

Macroscopic evaluation
After 1 month remnants of the scaffold were visible at the site of implantation in both groups (Figure 2B). The bladder wall appeared thinner at the implantation site. At 6 months the implantation site had the same appearance as the native bladder wall, and was only recognizable by the marking sutures, in both groups (Figure 2C). The native bladder had a normal appearance in both groups at both time points. No signs of stone formation were found. The kidneys appeared normal.

Histological evaluation
Native tissue of posterior bladder wall
At 1 month a normal urothelial layer, without ulceration or squamous dysplasia was seen in both groups (Figure 3A,B,F,G). The submucosa had a multilayered structure. Superficially a vimentin and α-SMA positive layer was seen, with a dense ECM. Underneath, a layer of less dense connective tissue was present, with blood vessels and an interrupted muscularis mucosae (Figure 3D,E,I,J). In both groups slightly more capillaries and granulocytes were visible compared to normal bladder tissue, suggesting a slight inflammatory response. The superficial part of the detrusor muscle showed atrophy and fibrosis in group 1 (Figure 3A), similar to experimental bladder exstrophy [25]; a normal detrusor muscle was seen in group 2 (Figure 3F). Elastic fibres were absent in group 1 (Figure 3C), and scarce in group 2 at this time point (Figure 3H).
At 6 months the urothelial layer was normal in both groups (Figure 3K,L,N,O), with a typical multi-layered appearance of the submucosa. The superficial part of the detrusor muscle still showed some atrophy in group 1, with more collagen between the muscle fibres, however this was less compared to 1 month (Figure 3K). Elastin was visible in both groups between the muscle fibres, arranged in groups of fibres, slightly more in group 2 (Figure 3M,P). Nerves were visible in both groups at both time points.

Regenerated bladder tissue
At 1 month, regeneration of the urothelial layer was incomplete in both groups (Figure 4A,B and Figure 4H,I). Ingrowth of urothelial cells occurred from the borders of the native tissue, and cell layers were gradually thinner towards the centre of the regenerated tissue at which urothelial cells were absent. In group 1, 2 out of 3 lambs

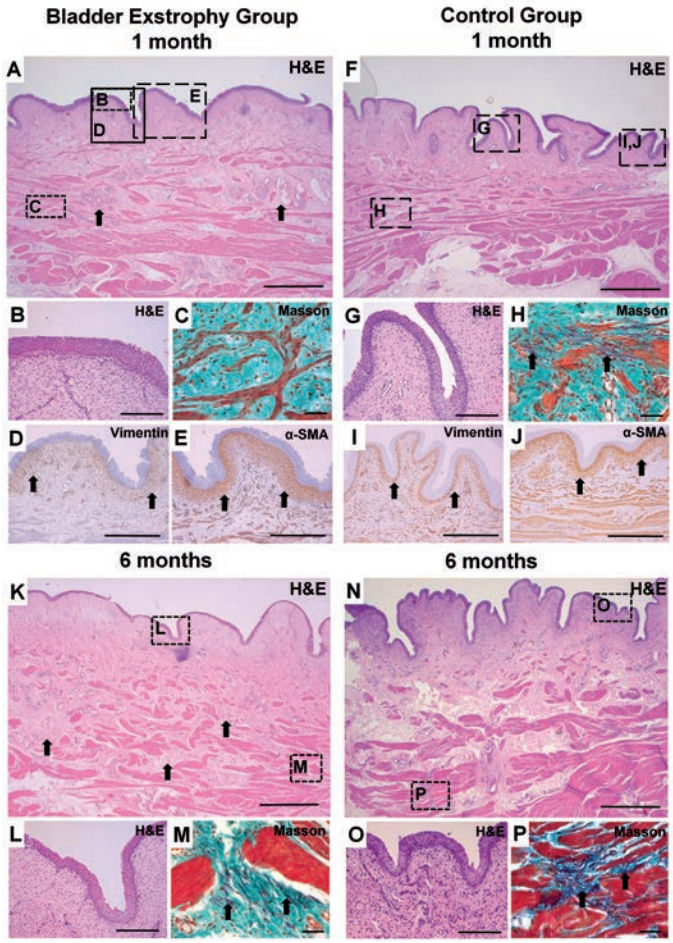

Figure 3. *Histology of the native posterior wall of the bladder at 1 month: Group 1 (bladder exstrophy group):* ***A.*** *overview of bladder tissue, note the atrophic and fibrotic inner layer of detrusor muscle (arrows), B-E are magnifications of the corresponding boxes in this overview,* ***B.*** *normal urothelial layer,* ***C.*** *no elastin visible,* ***D.*** *and* ***E.*** *vimentin and α-SMA staining showing multi-layered structure of submucosa (arrows).*
Group 2 (control group): ***F.*** *overview of bladder tissue, G-J are magnifications of the corresponding boxes in this overview,* ***G.*** *normal urothelial layer,* ***H.*** *elastic fibres visible (arrows),* ***I.*** *and* ***J.*** *vimentin and α-SMA staining showing multi-layered structure submucosa (arrows).*
Histology of the native posterior wall of the bladder at 6 months: Group 1: ***K.*** *overview of bladder tissue, note that the inner layer of detrusor muscle still shows atrophy and fibrosis (arrows), L-M are magnifications of the corresponding boxes in this overview* ***L.*** *normal urothelial layer,* ***M.*** *elastic fibres visible (blue) (arrows).*
Group 2: ***N.*** *overview of bladder tissue, O-P are magnifications of the corresponding boxes in this overview,* ***O.*** *normal urothelial layer,* ***P.*** *elastic fibres visible (arrows). Scale bar = 1 mm (A,F,K,N), 500 µm (D,E,I,J), 200 µm (B,G,L,O) or 50µm (C,H,M,P).*

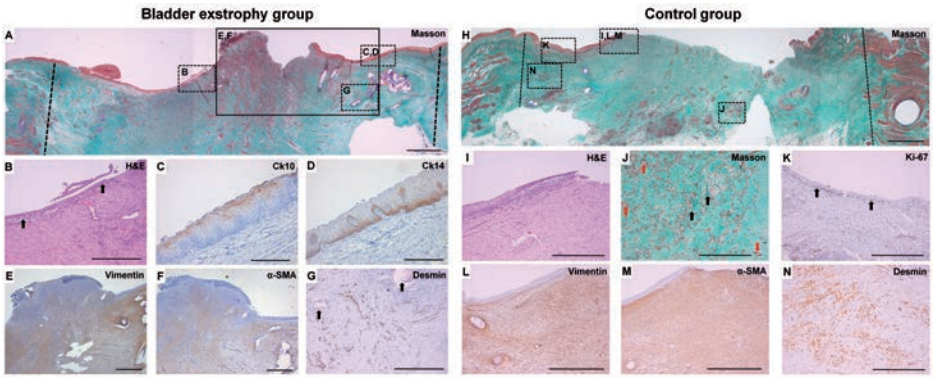

Figure 4. *Histology of generated tissue of group 1 at 1 month:* ***A.*** *overview, generated tissue between lines, purple marking sutures visible, B-G are magnifications of the corresponding boxes in this overview,* ***B.*** *newly generated urothelium (arrows) and connective tissue,* ***C.*** *and* ***D.*** *squamous dysplasia of urothelium at the border of generated tissue,* ***E.*** *abundant vimentin positive cells,* ***F.*** *abundant α-SMA positive cells,* ***G.*** *some smooth muscle cells (brown) (marking sutures at arrows). Histology of generated tissue of group 2 at 1 month:* ***H.*** *overview, generated tissue between lines, purple marking sutures visible, I-N are magnifications of the corresponding boxes in this overview,* ***I.*** *newly generated urothelium, underneath connective tissue,* ***J.*** *connective tissue and abundant neovascularisation (red arrows), remnants of scaffold (black arrows),* ***K.*** *proliferative cells (brown) (arrows),* ***L.*** *abundant vimentin positive cells,* ***M.*** *abundant α-SMA positive cells,* ***N.*** *some smooth muscle cells. Scale bar = 1 mm (A,E,F,H,L,M), 500 μm (B,G,I-K,N) or 200 μm (C,D).*

showed squamous differentiation of the urothelium at the border between the generated tissue and the native tissue (Figure 4A,C,D). No squamous differentiation was seen in group 2. Submucosal tissue consisted mainly of connective tissue with fibroblasts, myofibroblasts and deposited collagen (Figure 4A,B,E,F and Figure 4H-J,L,M). Neovascularisation was visible throughout the entire regenerated area in both groups (Figure 4J). Some remnants of the scaffold remained, mainly superficially at the bladder lumen (Figure 4H,J). Inflammatory cells were still apparent after 1 month, as part of the regeneration process, mainly consisting of granulocytes, and some lymphocytes, macrophages and giant cells. Ki-67 positive cells were largely located at the basal layer of the urothelium (Figure 4K). Smooth muscle cell ingrowth was present at the boundaries of the regenerated tissue (Figure 4G and Figure 4N). Elastic and nerve fibres were absent in both groups.

After 6 months the regenerated tissue showed a confluent urothelial layer in both groups (Figure 5A,B and Figure 5H,I). The urothelium appeared normal, i.e. was multilayered, had a basal layer and umbrella cells, without squamous differentiation. The submucosa was more organised than after 1 month, resembling the multi-layered structure of native tissue (Figure 5A,E,F and Figure 5H,L,M). Normal vascularisation was visible, including small capillaries underneath the urothelium, in both groups. Ki-67 staining was much lower compared to 1 month, implicating less proliferation at this time point. More ECM was visible in both groups compared to the

1 month time point, and the amount of myofibroblasts and fibroblast was decreased (Figure 5A,E,F and Figure 5H,L,M). In both groups smooth muscle cell ingrowth was present (Figure 5G and Figure 5N). These cells either formed fascicles or appeared as individual cells. Ingrowth occurred from the borders inwards, originating from the native detrusor muscle of the bladder. Some elastic fibres were found between the newly formed muscle fibres in both groups (Figure 5C and Figure 5J). The first nerve fibres were seen at the borders of the regenerated tissue (Figure 5D and Figure 5K).

Kidney tissue

No microscopic abnormalities were found in kidney tissue (data not shown).

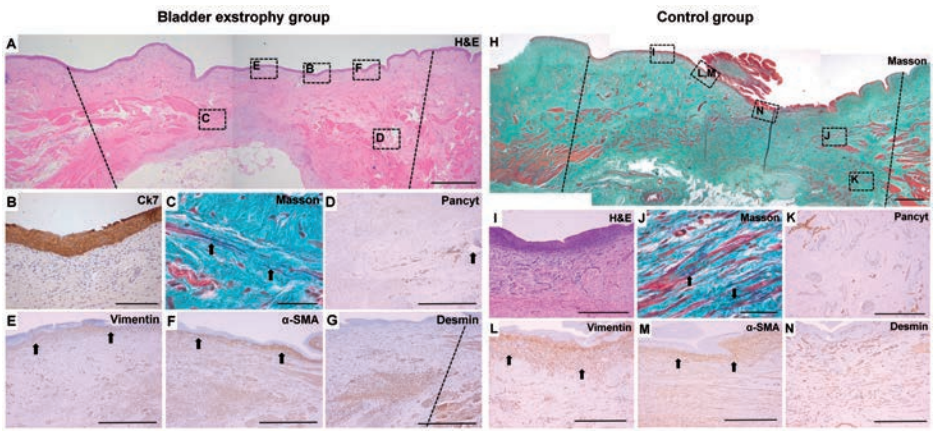

Figure 5. *Histology of generated tissue of group 1 at 6 months:* ***A.*** *overview, generated tissue between lines, B-G are magnifications of the corresponding boxes in this overview,* ***B.*** *ingrowth of muscle cells clearly visible, completely generated normal urothelium,* ***C.*** *elastic fibres visible (arrows),* ***D.*** *nerve fibres present near the border of generated tissue (brown) (marking suture at arrow) (pancytokeratin AE1/AE3),* ***E.*** *and* ***F.*** *multi-layered structure of submucosa (arrows), note the decrease of vimentin and α-SMA positive cells compared to 1 month post-surgery,* ***G.*** *smooth muscle cell ingrowth (line at border generated tissue).*
Histology of generated tissue of group 2 at 6 months: ***H.*** *overview, generated tissue between lines, ingrowth of muscle cells clearly visible, I-N are magnifications of the corresponding boxes in this overview,* ***I.*** *completely generated normal urothelium,* ***J.*** *elastic fibres visible (arrows),* ***K.*** *nerve fibres near the border of generated tissue (pancytokeratin AE1/AE3),* ***L.*** *and* ***M.*** *multi-layered structure of submucosa (arrows), note the decrease of vimentin and α-SMA positive cells compared to 1 month post-surgery,* ***N.*** *smooth muscle cell ingrowth. Scale bar = 1 mm (A,D-H,L-N), 500 µm (I,K), 200 µm (B) or 50 µm (C,J).*

Discussion

In this study we compared the regenerative capacity of diseased bladder to healthy bladder, using a highly porous collagen scaffold. We used a large (ovine) animal model for bladder exstrophy, which highly resemblances the bladder changes occurring in human bladder exstrophy [25]. Video urodynamics showed no difference in capacity or compliance of the bladders between both groups at 1 and 6 months. A trend of decreased compliance was seen in the bladder exstrophy group. However, this was not statistically significant due to large variations between animals and low number of animals in the groups due to unexpected animal loss. The decreased compliance was presumably caused by the fibrotic changes in the native bladder wall in these lambs. These changes in the bladder wall were seen immediately after birth in previous work [25], and partially persisted at least 1 month as shown in this study. Even after 6 months some atrophy and fibrosis of the detrusor muscle remained. No further abnormalities of the native bladder wall were found in group 1 when studied 1 or 6 months after reconstruction. This means that the bladder changes visible in our previous study, which highly resembled human bladder exstrophy [25], had partially resolved in this region of the bladder at these time points. Histological evaluation revealed no major differences between regeneration of bladder tissue in diseased and healthy bladders. Squamous differentiation of the urothelium was present at the borders between native and regenerated tissue 1 month after reconstruction in group 1. Squamous differentiation is often seen in neonates with bladder exstrophy, and was also visible in the previous study with the current animal model [25]. This might suggest the presence of urothelial changes in regenerated tissue of diseased bladders at early time points. However, after 6 months the urothelium of the regenerated tissue appeared normal, implicating a reversible effect.

Whether 'diseased' bladders, like for instance in bladder exstrophy or neuropathic bladders, are able to generate normal bladder tissue when using tissue engineering is a major concern. *In vitro* studies of isolated urothelial cells from diseased bladders suggested impaired proliferation and differentiation [12,13]. In addition, other *in vitro* studies indicated that cultured smooth muscle cells from patients with bladder exstrophy and neuropathic bladders also have altered characteristics [11,14-20]. However, the use of smooth muscle cells as a cell source for tissue engineering in a neuropathic bladder is questionable. A non-contractile augmented bladder segment may be more useful than a bladder part which may function similar to the native neuropathic bladder. *In vivo* studies in a diseased animal model were performed by Zhang *et al*., in which 90% of the bladder was resected and after 1 month an augmentation with a small-intestinal-submucosa (SIS) scaffold was performed [21], and by Akbal *et al.*, who created a high-pressure bladder which was later augmented with acellular dermal biomatrix [22]. These studies showed impaired regeneration of bladder tissue when using an unseeded collagen scaffold. Although a normal urothelial layer was found, the underlying tissue was more fibrotic and smooth

muscle formation was impaired. However, one could comment on the first study that the resection of a very large bladder segment created a highly challenging model, perhaps with even worse characteristics than in human diseased bladders. In the other study the bladder catheter was left in place during the whole study period, thereby hampering bladder cycling, which is an important factor for normal bladder development and regeneration [6,17,32]. In contrast to the findings by Zhang *et al.* and Akbal *et al.* our study suggests that diseased bladder tissue can maintain its capacity to regenerate new tissue, with the same characteristics as the native bladder. The contradicting results may not be caused by the tissue-engineered solution but by the degree of severity of damage of the diseased bladder model.

In this study we used a highly porous type I collagen scaffold with proven regenerative potential [25,29,33]. This carrier material was chosen based on the predominant occurrence in the normal bladder, low antigenicity, and good biodegradability and cell-binding capacity. We used a low collagen content scaffold (0.67%) to increase cell ingrowth and minimize antigenicity, while keeping the handling properties for the surgeon intact [34]. A scaffold with a diameter of 32 mm was used because our current goal was to study the intrinsic capacity of diseased bladder to regenerate tissue, which was inherently accompanied by the possibility that the surface would be too small for clinically significant augmentation. This resulted in good quality of regenerated tissue meaning: complete regeneration of the bladder wall, including the formation of smooth muscle cell bundles, mucosa, vessels, and a neuronal network, as defined by Zhang *et al.* [21]. Nevertheless, new strategies may be needed when larger augmentations are necessary to obtain more capacity and better compliance, particularly in severely diseased bladders. Incorporation of autologous cells in scaffolds has been shown to be a good option. However, as previous studies showed, cultured cells of diseased bladders may lose their phenotype and capacity to proliferate and differentiate *in vitro.* Perhaps optimization of culture conditions could reverse these changes in diseased cells [12,17]. Alternatively, the use of stem cells or alternative donor tissue, such as buccal mucosa for epithelial cells, might be optional [12]. However, in this neonatal model a source for bladder cells is not available, except for highly challenging techniques as performing a prenatal biopsy of the bladder to receive cells [24] or the use of amniocytes [35]. Another approach to enhance cell migration and proliferation and neovascularisation is the use of growth factors, which are capable to attract cells from the surrounding tissue [29,33]. This technique has the advantage of delivering a product which is 'off-the-shelf' available, and may not need the time- and cost-consuming techniques in which cells are used.

In conclusion, we showed that bladder tissue engineering with a highly porous collagen scaffold is possible in a diseased model. Regeneration of the bladder was comparable to regeneration in healthy bladder, when using an animal model for bladder exstrophy, and resulted in tissue of good quality. Improvements of the technique may still be needed for larger augmentations or more severely diseased bladders.

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Chapter 4

Bladder Regeneration using a Smart Acellular Scaffold with Growth Factors

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Submitted

Abstract

Background: Tissue engineering may become an alternative to current bladder augmentation techniques. Large scaffolds are needed for clinically significant augmentation, but can result in fibrosis and graft shrinkage.

Objective: To investigate whether smart acellular collagen-heparin scaffolds with growth factors VEGF, FGF2 and HB-EGF enhances bladder tissue regeneration and bladder capacity in a large animal model of diseased bladder.

Design, setting, and participants: Scaffolds of bovine type I collagen loaded with heparin and VEGF, FGF2 and HB-EGF measuring 3.2 cm in diameter were prepared. In 23 fetal sheep a bladder exstrophy was surgically created at 79 days' gestation. Lambs were born at full term.

Interventions: One week after birth the bladder lesion was reconstructed by primary closure (PC-group) or using a collagen-heparin scaffold with (COLGF-group) or a collagen scaffold without growth factors (COL-group).

Outcome measurements and statistical analysis: Functional (video urodynamics) and histological evaluation was performed 1 and 6 months after bladder repair. One-way ANOVA was used, followed by the LSD post hoc test.

Results and limitations: Survival rate was 57%, 13 lambs completed the study. Cystograms were normal in all animals, except for low grade reflux in all groups. Urodynamics showed no statistically significant differences in bladder capacity and compliance between groups. Histological evaluation at 1 month revealed increased urothelium formation, improved angiogenesis and enhanced ingrowth of smooth muscle cells in the COLGF-group compared to the COL-group. At 6 months improved smooth muscle cell ingrowth was found in the COLGF-group compared to the COL-group; both scaffold groups showed normal urothelial lining and standard extracellular matrix development.

Conclusions: Bladder regeneration using a collagen-heparin scaffold with VEGF, FGF2 and HB-EGF improved angiogenesis, urothelial and smooth muscle cell ingrowth in a large animal model of diseased bladder. Larger growth factor loaded constructs need to be tested to reach clinically significant augmentation.

Introduction

Gastrointestinal tissues are most often used for bladder augmentation. However, this is not always sufficiently available, and its use can lead to metabolic disturbances, infections, excessive mucus production, stone formation, perforation and even malignancies [1-4]. Tissue engineering techniques aim to repair or replace damaged or removed organs by regeneration. Studies in various animal models have shown that tissue-engineered constructs can regenerate bladder tissue, including urothelium, smooth muscle, vessels and nerve fibres [5-10].The first clinical trials showed the feasibility of tissue engineering of the human bladder, with a substantial improvement in bladder capacity [10,11]. Nevertheless, improvements are needed to enhance clinical outcome [6,7,10,11].

Regeneration in large constructs is hampered by lack of oxygen and nutrition delivery to the cells and inadequate removal of waste products [12,13], since the diffusion distance from the supplying blood vessel is approximately 150-200 μm [13]. Therefore angiogenesis needs to be improved. Growth factors (GFs) are involved in proliferation, migration and differentiation of several cell types, and inclusion in large grafts may assist in quicker and better acceptance. GF addition to scaffolds resulted in improved bladder regeneration in small animals [14-18].

Our objective was to investigate the effect of incorporation of GFs in a collagen-heparin scaffold on bladder tissue regeneration and functionality in a large animal model for diseased bladder to permit better comparison with humans [19-21]. We previously showed that an exstrophied bladder created in fetal lambs shows remarkable similarities with human bladder exstrophy when studied directly after birth [21]. Vascular endothelial growth factor (VEGF), an important factor in angiogenesis, combined with fibroblast growth factor 2 (FGF2) enhances blood vessel formation and maturation [12]. These GFs were bound to collagen scaffolds loaded with heparin, together with heparin-binding epidermal growth factor (HB-EGF) which is known to play a role in urothelial regeneration [22-24]. Outcomes of reconstruction of an experimental bladder exstrophy using this scaffold were compared with outcomes in a historical control group in which a scaffold without GFs was used [25] and a group that was reconstructed without a scaffold.

Materials and methods

Preparation and characterization of scaffolds

Round collagen scaffolds with a diameter of 3.2 cm were prepared from insoluble bovine type I collagen fibrils and incorporated with heparin as previously described [26]. Scaffolds were disinfected in 70% ethanol followed by washings with sterile PBS. Collagen-heparin scaffolds were aseptically incubated with fibroblast growth factor 2 (FGF2), vascular endothelial growth factor 165 (VEGF) and heparin-binding epidermal growth factor (HB-EGF) (all human recombinant; R&D Systems, Minneapolis, MN, USA). First, scaffolds were incubated in 7 ml PBS containing 3.5 µg/ml FGF2 for 1h. Next, VEGF and HB-EGF, each 3.5 µg/ml PBS, were added and incubated overnight at 22°C. Finally, scaffolds were washed in PBS and used immediately.

Degree of crosslinking was determined spectrophotometrically from the number of primary amine groups using 2,4,6-trinitrobenzene sulfonic acid [27,28]. Heparin content was determined by a hexosamine assay with p-dimethylamino-benzaldehyde, using a standard curve of heparin [29]. GF content was assessed using Western blot analysis [22]. Ultrastructure of the scaffolds was visualized by scanning electron microscopy (SEM) using a JEOL JSM-6310 SEM apparatus operating at 15 kV after gold-coating the specimens.

Surgical procedures

This study was approved by the Ethical Committee on Animal Research of the Radboud university medical center.

Prenatal operations

Twenty-three pregnant sheep (Dutch Texel breed) and fetuses were operated at 79 days' gestation (full term 140-147 days). The fetal operation was earlier described [21], resulting in an exstrophied bladder measuring approximately 1.5 x 1 cm (Figure 1A). Parturition was induced at 140 days' gestation as described [25], resulting in vaginal delivery.

Postnatal operations

Surviving lambs were operated one week after birth (Figure 1B). Perioperative management was performed as described [25]. The bladder plate was surgically detached from the abdominal wall, and 3-4 mm of the transition zone between the bladder plate and the abdominal wall was removed. Lambs were randomly assigned to either the primary closure-group (PC-group) or the growth factor-group (COLGF-group). In the PC-group the bladder was closed in two layers using a 6-0 poliglecaprone (Monocryl®, Ethicon Inc.) running suture. In the COLGF-group the scaffold was sutured onto the bladder plate using 6-0 poliglecaprone running sutures (Figure 1C). Four 6-0 polypropylene (Prolene®, Ethicon Inc.) nonresorbable

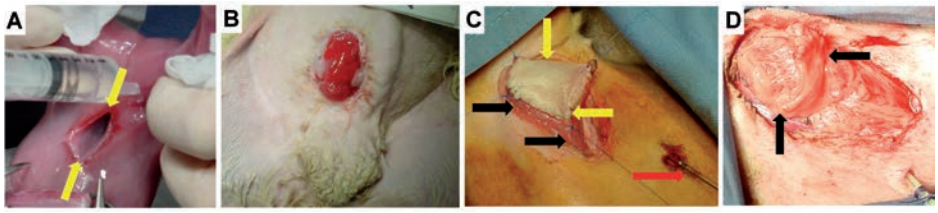

Figure 1. *Surgical procedures.* ***A.*** *Prenatal operation: fetal bladder was opened (between arrows) and sutured to the abdominal wall.* ***B.*** *Postnatal macroscopic result.* ***C.*** *Postnatal operation: collagen scaffold (between yellow arrows) sutured to bladder plate (black arrows). Suprapubic catheter (red arrow).* ***D.*** *Scaffold covered with omentum (black arrows).*

marking sutures were placed at the edges of the scaffold. A 5Fr suprapubic catheter (Cystofix®, B. Braun) was placed and fixed to the bladder using 6-0 poliglecaprone. To cover the collagen scaffold and to promote angiogenesis, the omentum was sutured onto the bladder with 6-0 poliglecaprone (Figure 1D). The abdominal wall was closed using 2-0 polyglactin (Vicryl®, Ethicon Inc.) interrupted sutures. The suprapubic catheter was fixed to the skin using 2-0 polyglactin sutures, and was removed after 3 weeks.

Neonatal outcome and evaluation

Animals were evaluated randomly after 1 or 6 months. Video urodynamic evaluation was performed as described [25]. One to three representative evaluations were performed in each lamb. Thereafter the lambs were sacrificed as described [25]. The bladder was removed and inspected. Kidneys were examined for signs of infection, hydronephrosis, or stone formation.

Histological staining

Tissue samples were obtained of the regenerated tissue (COLGF-group) and the scar tissue (PC-group), fixed in 4% (v/v) buffered formalin and paraffin-embedded. Sections (4 µm) were cut and stained with hematoxylin & eosin (H&E) and Masson's trichrome staining. For immunohistochemistry, sections were deparaffinized in xylene, followed by graded series of ethanol and re-hydration in PBS. Slides were immersed in 3% (v/v) H_2O_2/PBS for 30 min at 22°C to block endogenous peroxidase. Antigen retrieval methods and primary antibodies are shown in Table 1. Slides were pre-incubated with 5% goat serum, incubated with the antibody of interest, washed and incubated with poly-HRP-anti Mouse IgG (Immunologic, Duiven, the Netherlands). After a PBS wash the slides were incubated in PowerVision DAB (Immunologic) for 10 min at 22°C, washed in tap water and counterstained with Mayer's haematoxylin (Fluka Chemie, Buchs, Switzerland). Renal tissue of all lambs was processed and examined for inflammatory changes or dilated nephrons by H&E staining.

Table 1. *Antibodies used for immunohistological evaluation*

Antigen	Antibody	Source	Dilution	Antigen retrieval
CK 7	RCK105	MUbio BV	1:10	A
CK 10	RKSE-60	MUbio BV	1:5	A
CK 14	LL002	Thermo Fisher scientific	1:100	A
Pancytokeratin AE1/AE3	AE1/AE3	Thermo Fisher scientific	1:800	B
Vimentin	V-9	BioGenex	1:2000	C
α-SMA	1A4	Sigma-Aldrich	1:15000	C
Desmin	[33]	BioGenex	1:200	C
Smoothelin	R4A	Santa Cruz Biotechnology	1:150	A

A: Heat mediated in sodium citrate buffer (10 mM; pH 6.0; 10 min; 100°C); B: with 0.1% pronase (30 min at room temperature); C: without antigen retrieval

Statistical analysis

Data analysis was performed with IBM SPSS 20, using the one-way ANOVA test, followed by the LSD post hoc test. $P < 0.05$ was considered statistically significant. $P < 0.05$ was considered statistically significant.

Results

Scaffolds

The collagen scaffolds consisted of homogenously distributed honeycomb-like pores with an average size of 100-150 µm. Percentages of amine groups used in the crosslinking process were 31 ± 8% for collagen scaffolds and 31 ± 4% for heparinised collagen scaffolds. The percentage of heparin bound to heparinised collagen scaffolds was 13 ± 2%. Amounts of GFs bound per mg collagen-heparin scaffold were 0.31 ± 0.15 µg for VEGF, 0.72 ± 0.32 µg for FGF2, and 0.46 ± 0.24 µg for HB-EGF.

Animal surgery

Survival rate and complications of the operated animals are shown in Table 2.
To decrease harm to and numbers of animals, the groups were compared to a historical control group of lambs (COL-group), described in previous work [25], that received the same prenatal operation during the same period and underwent reconstruction with the same collagen scaffold without heparin and GFs.

Functional evaluation

All lambs voided normally, without signs of incontinence or urinary tract infection. None of the lambs showed detrusor overactivity on urodynamic evaluation. Urodynamic data are shown in Table 3. Capacity increased between 1 and 6 months in the PC-group ($p = 0.04$) and the COL-group ($p=0.02$), but the increase was not statistically significant in the COLGF-group ($p=0.11$). Compliance increased over time in the PC-group ($p=0.02$). The 3 groups did not statistically significantly differ in capacity and compliance at both time points. Cystograms showed no structural

abnormalities of the bladder, e.g. no diverticulum or leakage (Figure 2A). In almost all lambs low grade reflux was seen near maximal intravesical pressure.

Table 2. *Survival and complications*

	PC-group	COLGF-group	Complications
Operated fetuses		23	Fetuses: 5 intra-uterine demise 1 died during delivery
Operated lambs		17 (74%)	Lambs: PC-group: 1 died during operation
Overall survival		13 (57%)	1 sacrificed due to bladder outlet obstruction
Results 1 month	3	3	COLGF-group: 1 died immediately post-operative 1 sacrificed due to abdominal infection
Results 6 months	3	4	

Table 3. *Results of urodynamics performed in lambs*

	Capacity (ml)				Compliance (ml/cmH_2O)		
1 month							
Lamb	PC-group	COL-group	COLGF-group	Lamb	PC-group	COL-group	COLGF-group
1	40	50	45	1	1.2	2.6	1.4
2	19	14	43	2	0.5	0.5	3.7
3	19	NA	NA	3	0.7	NA	NA
Mean	26	32	44		0.8	1.5	2.6
SD	12	25	1		0.3	1.4	1.6
6 months							
Lamb	PC-group	COL-group	COLGF-group	Lamb	PC-group	COL-group	COLGF-group
1	50	82	32	1	1.7	2.1	1.4
2	51	73	100	2	5.0	2.2	3.6
3	96	70	87	3	2.6	2.1	2.7
4		94	80	4		2.8	2.2
Mean	66	80	75		3.1	2.3	2.4
SD	26	11	29		1.7	0.3	0.9

NA = not available (leakage next to catheter during urodynamics); SD = standard deviation

Macroscopic evaluation

After 1 month, scaffold remnants were visible at the site of implantation in both scaffold-groups (Figure 2B). The bladder wall appeared thinner at the implantation site. After 6 months, the implantation site had the same appearance as the native bladder wall, and was only recognizable by the marking sutures (Figure 2C). A small scar was visible in the PC-group after 1 month, that was unrecognizable after 6 months. The native bladder had a normal appearance in all groups at both time points, without signs of stone formation. The kidneys appeared normal.

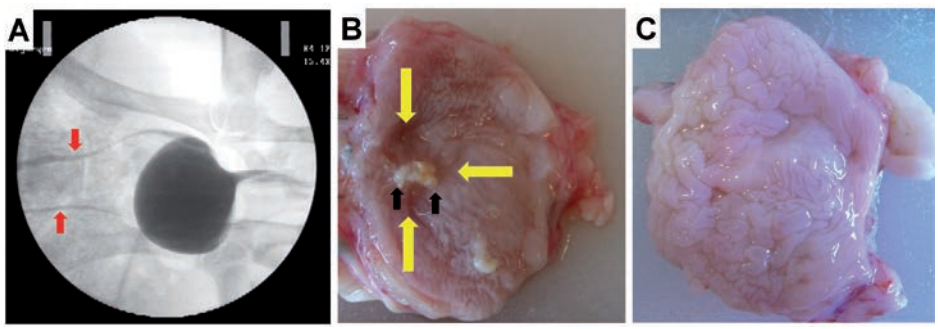

Figure 2. A. *Cystogram showing no abnormalities except bilateral reflux (red arrows).* ***B.*** *Macroscopic view inside of bladder at 1 month. Regenerated tissue between yellow arrows. Remnant of scaffold visible at black arrows.* ***C.*** *Macroscopic view inside of bladder at 6 months. No differences seen between regenerated and native bladder.*

Histological evaluation

Primary closure-group

One month post-reconstruction the urothelial layer was still incomplete. Scar tissue was present in the wound, composed of deposited collagen, fibroblasts and myofibroblasts. Six months post-reconstruction a confluent, normal appearing multilayered lining of urothelial cells was seen, containing a basal layer and umbrella cells. Scar tissue had resolved and architecture of the tissue was normal, including smooth muscle formation (data not shown).

Regenerated bladder tissue in scaffold-groups

One month post-reconstruction the urothelial layer was incomplete in the COL-group, and gradually thinner towards the centre of the regenerated tissue where urothelial cells were absent (Figure 3A,B). In contrast, the urothelial layer was completely regenerated in the COLGF-group (Figure 4A,B,C). The urothelium appeared normal, was multilayered, and contained a basal layer and umbrella cells. Squamous differentiation of the native urothelium was observed in 2 lambs of the COLGF-group. In one of these lambs the adjacent regenerated urothelium also showed squamous differentiation (Figure 4D), in the other lamb the adjacent

urothelium was normal (Figure 4E). Similarly, 2 lambs of the COL-group also showed squamous differentiation of the urothelium at the edges of regenerated tissue (Figure 3A,C,D) [25].
Submucosal tissue was similar in all animals and mainly consisted of connective tissue with fibroblasts, myofibroblasts and deposited collagen (Figure 3A,E,F and 4A,F-H). Angiogenesis was visible throughout the entire regenerated area and much more profound in the COLGF-group (compare Figure 3A,B and 4A,B). Blood vessels were mature, evidenced by α-SMA-positive cellular linings and the presence of erythrocytes (Figure 3A,B,F and 4A,B,H). Some scaffold remnants remained, mainly superficially at the bladder lumen, but also deeper in the regenerated tissue (Figure 4F). Inflammatory cells were visible, as part of the regeneration process after 1 month, mostly located under the urothelium. These mainly consisted of granulocytes, and some lymphocytes, macrophages and giant cells. Smooth muscle cell (SMC) ingrowth was present at the boundaries in 2 lambs, and through the entire regenerated tissue in 1 lamb in the COLGF-group, clearly more profound compared to the COL-group (compare Figure 3G with Figure 4I). Elastic and nerve fibres were absent in both groups.
After 6 months the regenerated tissue showed a confluent, well differentiated, urothelial layer, without squamous differentiation, in both groups (Figure 5A,B and 6A,B). The organisation of the submucosa was improved compared to 1 month post-reconstruction, resembling the multi-layered structure of native bladder (Figure 5E,F and 6E,F). Vascularisation was normal. ECM was increased compared to 1 month post-reconstruction, and the number of myofibroblasts and fibroblast was decreased (Figure 5E,F and 6E,F). SMC ingrowth was present throughout the entire regenerated tissue, appearing as fascicles or separate cells, and slightly more profound in the COLGF-group (compare Figure 5G and 6G). SMC ingrowth occurred from the borders inwards, originating from the native detrusor muscle. Elastic fibres were found between the newly formed muscle fibres (Figure 5C and 6C). Nerve fibres were seen at the borders of the tissue (Figure 5D and 6D).

Kidney tissue
No abnormalities were found in kidney tissue (data not shown).

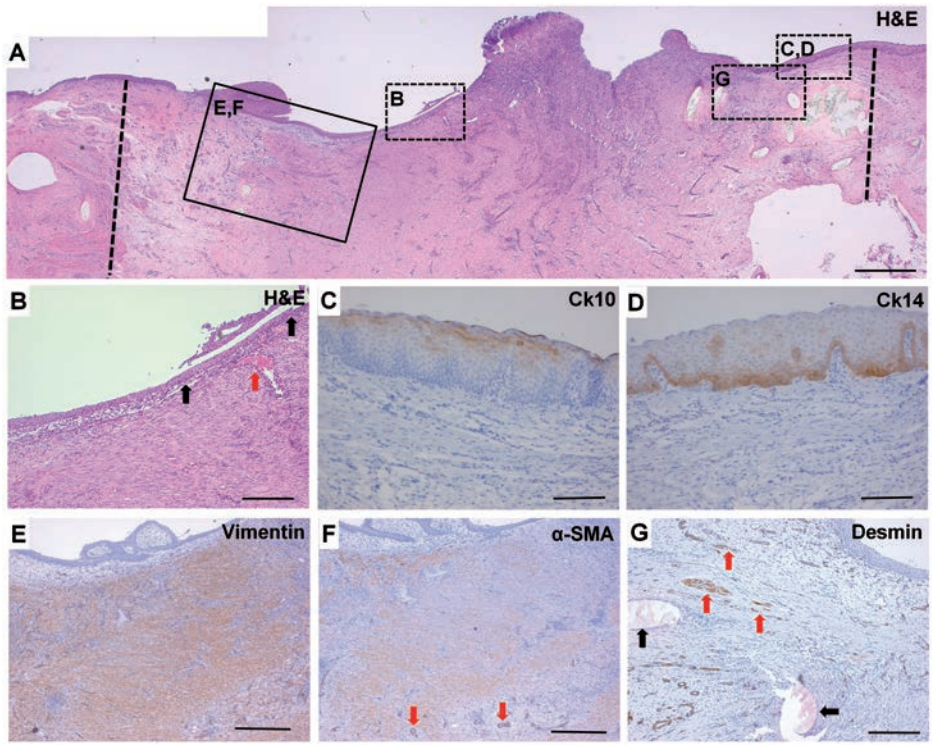

Figure 3. *Histology of regenerated tissue of the COL-group at 1 month.* ***A.*** *Overview, regenerated tissue between dotted lines, grey marking sutures visible. B-G are magnifications of the corresponding boxes in this overview.* ***B.*** *Regenerated urothelium (black arrows), underneath connective tissue. Mature blood vessel with erythrocytes at red arrow.* ***C*** *and* ***D.*** *Squamous dysplasia of urothelium at the border of regenerated tissue.* ***E.*** *Submucosal connective tissue with abundant vimentin-positive cells.* ***F.*** *Submucosal connective tissue with abundant α-SMA-positive cells. Blood vessels at red arrows.* ***G.*** *Some ingrowth of smooth muscle cells (brown) (red arrows) at the border of regenerated tissue. Marking sutures at arrows. Scale bar = 1 mm (A), 500 µm (B,E-G) or 100 µm (C,D).*

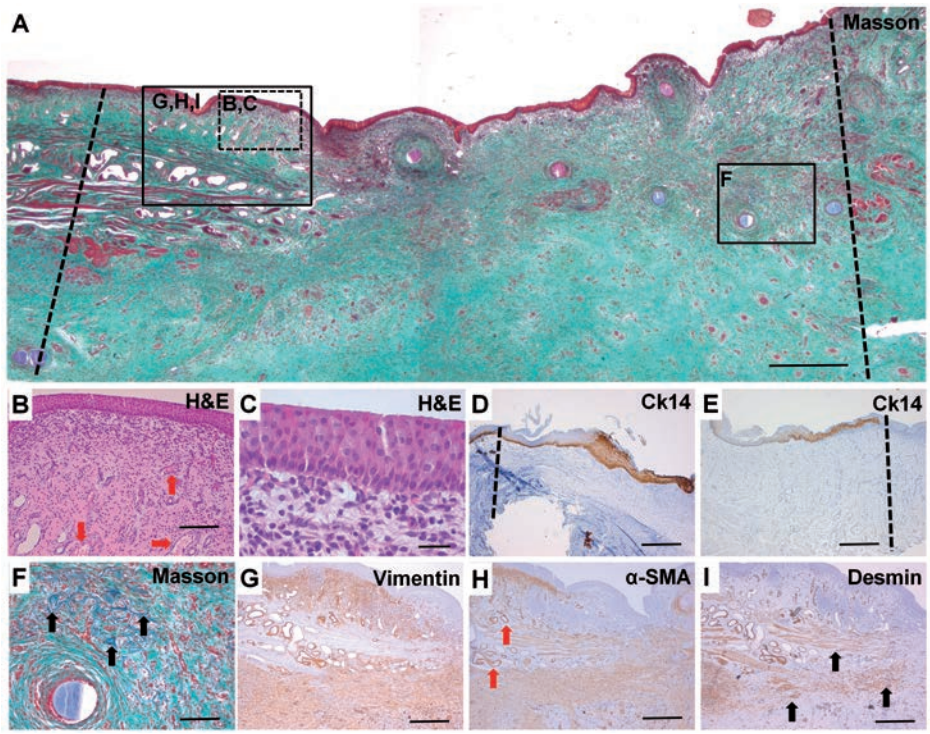

Figure 4. *Histology of regenerated tissue of the COLGF-group at 1 month.* ***A.*** *Overview, regenerated tissue between dotted lines, blue marking sutures visible. B,C,F-I are magnifications of the corresponding boxes in this overview, D and E are from other lambs.* ***B.*** *Regenerated urothelium, submucosa with mature blood vessels (red arrows).* ***C.*** *Normal appearing urothelium.* ***D*** *and* ***E.*** *Squamous dysplasia of urothelium at the border of regenerated tissue (regenerated tissue at right side of dotted line).* ***F.*** *Remnants of scaffold (blue) (black arrows).* ***G.*** *Abundant vimentin positive cells in submucosal tissue.* ***H.*** *Abundant α-SMA positive cells and mature blood vessels (red arrows).* ***I.*** *Smooth muscle cell (brown) (black arrows) ingrowth. Scale bar = 1 mm (A), 500 µm (D,E,G-I), 100 µm (B,F) or 25 µm (C).*

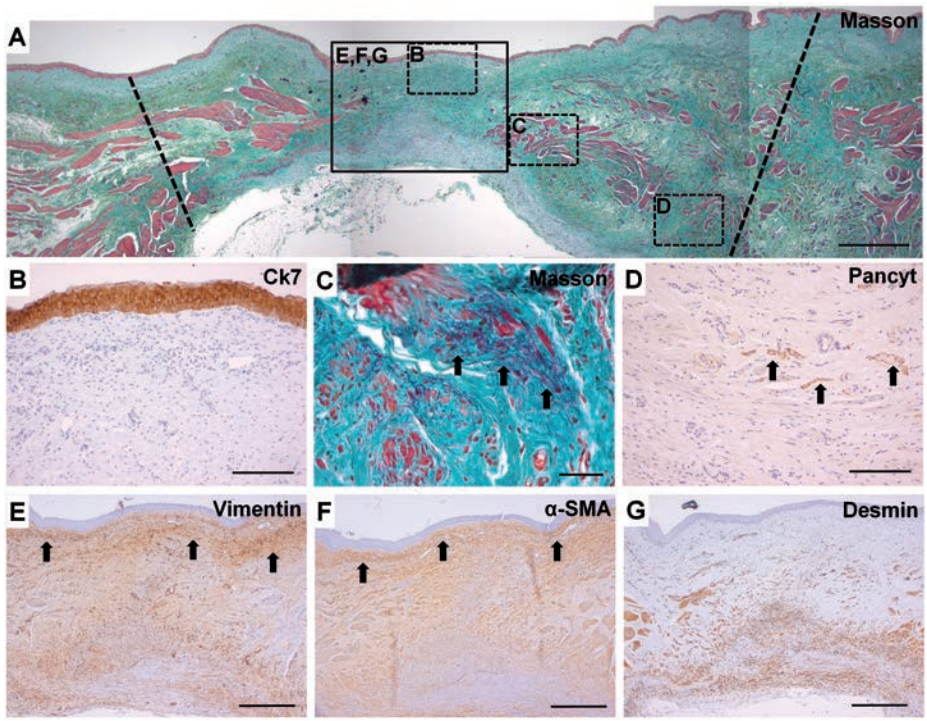

Figure 5. *Histology of regenerated tissue of the COL-group at 6 months.* ***A.*** *Overview, regenerated tissue between dotted lines, ingrowth of muscle cells (red) clearly visible. B-G are magnifications of the corresponding boxes in this overview.* ***B.*** *Normal regenerated urothelium.* ***C.*** *Elastic fibres visible (arrows).* ***D.*** *Nerve fibres near the border of regenerated tissue.* ***E*** *and* ***F.*** *Multi-layered structure of submucosa (arrows), note the decrease of vimentin and α-SMA positive cells compared to 1 month post surgery.* ***G.*** *Smooth muscle cell ingrowth (brown). Scale bar = 1 mm (A), 500 µm (E-G), 200 µm (B,D) or 50 µm (C).*

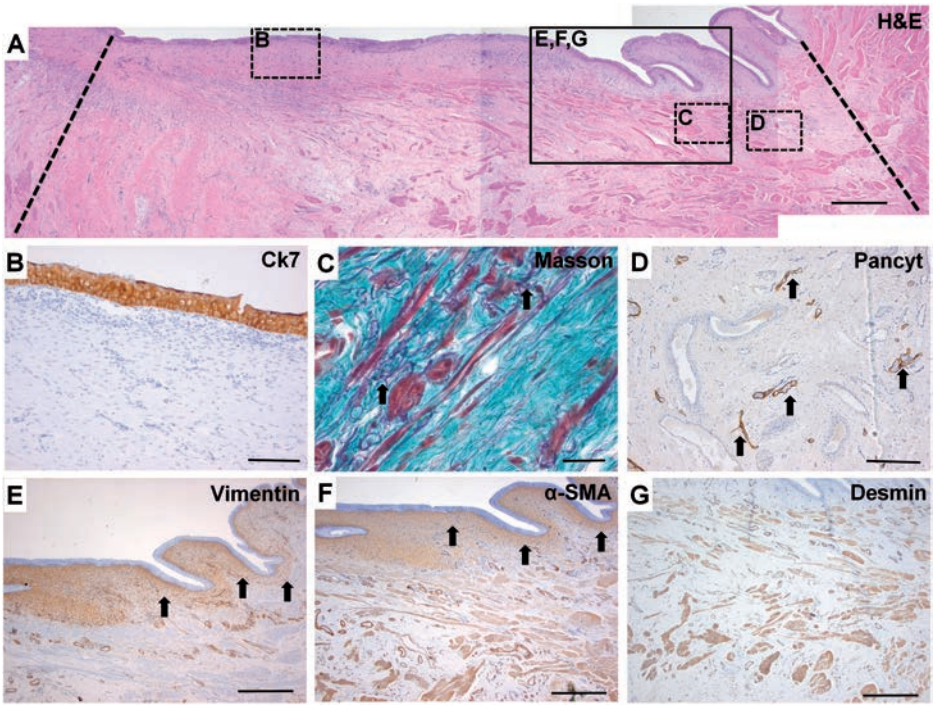

Figure 6. *Histology of regenerated tissue of the COLGF-group at 6 months.* ***A.*** *Overview, regenerated tissue between dotted lines. B-G are magnifications of the corresponding boxes in this overview.* ***B.*** *Normal urothelium.* ***C.*** *Elastic fibres (blue) at black arrows.* ***D.*** *Nerve fibres (brown perineurium) present near the border of regenerated tissue.* ***E*** *and* ***F.*** *Multi-layered structure of submucosa (arrows), note the decrease of vimentin and α-SMA positive cells compared to 1 month post surgery.* ***G.*** *Smooth muscle cell ingrowth through entire regenerated tissue. Scale bar = 1 mm (A), 500 µm (E-G), 250 µm (D), 100 µm (B) or 25 µm (C).*

Discussion

In this study we evaluated regeneration of bladder tissue in a large animal model for bladder exstrophy, using a highly porous collagen-heparin scaffold loaded with GFs (COLGF-group). This model appears to closely mimic the human circumstances in which a bladder is diseased before repair is attempted, which might influence the regenerative process [25]. The COLGF-group was compared to a historical control group in which a collagen scaffold without GFs was used (COL-group) [25], and a group in which the bladder was closed without using a scaffold (PC-group). The addition of GFs clearly improved ingrowth of urothelial cells. This effect may be contributed to the stimulatory effect of HB-EGF, which plays an important role in urothelial cell proliferation, migration, differentiation and repair [22-24]. Squamous dysplasia is often found in neonates with bladder exstrophy [21] and may implicate an adverse effect of the diseased bladder on urothelial regeneration. The squamous dysplasia of the regenerated urothelium observed in some lambs 1 month post-reconstruction completely disappeared after 6 months, suggesting that this process is reversible.

Angiogenesis was more profound in the COLGF-group compared to the COL-group at 1 month, probably by the addition of VEGF and FGF2. Combining VEGF and FGF2 enhances blood vessel formation and maturation [12], resulting in an earlier onset of vasculature network formation, hereby reducing hypoxic conditions and improving tissue regeneration. The improved angiogenesis may have enhanced regeneration of other tissue components, such as the formation of the urothelial layer and perhaps also improved ingrowth of SMCs. Additionally, SMC ingrowth may have been influenced by the incorporation of FGF2 and HB-EGF, which induce proliferation, migration and differentiation of bladder SMCs [30-35].

This is the first study in which a scaffold with this combination of GFs was used in a large animal model to regenerate bladder tissue. Moreover, high concentrations of GFs were bound to the scaffolds through the use of heparin, that also stabilises GFs and protects them against proteolytic degradation, creating a sustained release system [36,37]. In other studies on bladder regeneration much lower amounts of GFs were used [14-18,38]. First, studies performed in rats showed promising results [14-16]. Thereafter, two studies were performed in rabbits and one in pigs, in which a bladder acellular matrix (BAM) incorporated with VEGF [17,38] or VEGF combined with platelet-derived growth factor BB (PDGF-BB) was used [18]. The use of GFs showed improved regeneration [17,18,38] and capacity [18]. Comparison to our study is difficult because the concentrations of GFs incorporated in the scaffold were very low [17,38] or not mentioned [18]. Clearly, the best combination and concentration of incorporated GFs still needs to be defined.

The improved regeneration of bladder tissue did not lead to statistically significant improved urodynamic outcome. A trend towards increased capacity and higher compliance was seen when using a scaffold, both at 1 month and at 6 months.

However, the sample sizes preclude drawing conclusions on this effect.
We used a scaffold of 3.2 cm in diameter, which was approximately 30% of the surface of the bladder at time of reconstruction, because our goal was to test whether growth factors improved regeneration. Larger scaffolds would be needed to reach clinically significant augmentation of the bladder. An acellular scaffold could result in central fibrosis and graft shrinkage [8,9]. Although incorporation of autologous urothelial and smooth muscle cells may overcome this problem, this requires time- and cost-consuming harvesting, culturing and seeding of cells. Furthermore, cells from diseased bladders may behave dissimilar to normal cells, making their use for tissue engineering questionable [25]. The use of GFs incorporated in an acellular scaffold provides for a product which is 'off-the-shelf' available for the surgeon and may overcome these problems.

Conclusions

Bladder tissue engineering with a highly porous collagen scaffold loaded with VEGF, FGF2 and HB-EGF enhanced tissue regeneration compared to a scaffold without GFs, when used in a large animal model for diseased bladder. This resulted in tissue of good quality involving all layers of the bladder. Nevertheless, larger constructs need to be tested for the ability to reach clinically significant augmentation.

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Chapter 5

Tissue Engineered Tubular Construct for Urinary Diversion in a Preclinical Porcine Model

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Journal of Urology 2012;188:653-60

Abstract

Purpose: The ileal conduit has been considered the gold standard urinary diversion for patients with bladder cancer and pediatric patients. Complications are mainly related to the use of gastrointestinal tissue. Tissue engineering may be the technical platform on which to develop alternatives to gastrointestinal tissue. We developed a collagen-polymer conduit and evaluated its applicability for urinary diversion in pigs.

Materials and Methods: Tubular constructs 12 cm long and 15 mm in diameter were prepared from bovine type I collagen and Vypro® II synthetic polymer mesh. Characterized tubes were sterilized, seeded with and without primary porcine bladder urothelial cells, and implanted as an incontinent urostomy using the right ureter in 10 female Landrace pigs. At 1 month the newly formed tissue structure was functionally and microscopically evaluated by loopogram and immunohistochemistry, respectively.

Results: The survival rate was 80% with 1 related and 1 unrelated death. By 1 month the collagen was resorbed and a retroperitoneal tunnel had formed that withstood 40 cm H_2O water pressure. In 5 cases the tunnel functioned as a urostomy. Histological analysis revealed a moderate immune response, neovascularization and urothelial cells in the construct lumen. The polymer mesh provoked fibroblast deposition and tissue contraction. No major differences were observed between cellular and acellular constructs.

Conclusions: After implanting the tubular constructs a retroperitoneal tunnel was formed that functioned as a urinary conduit in most cases. Improved large tubular scaffolds may generate alternatives to gastrointestinal tissue for urinary diversion.

Introduction

The ureteroileal cutaneous conduit or urostomy has been the primary technique for incontinent urinary diversion for more than 50 years [1]. Despite complication risks about 33% of urologists choose this technique [2]. The complication rate is between 20% and 56% within the first weeks after surgery, which can increase to 28% to 81% after 1 month [3,4]. Early complications are usually related to bowel resection and anastomosis while long-term complications are more stoma related [5]. Bowel related complications can include anastomotic leakage, enteric fistula, bowel obstruction and prolonged ileus.

Thus, it is desirable to prevent gastrointestinal tissue (GIT) use and create a urinary conduit from an artificial construct through which urine can be diverted. Preoperative care would be less intensive and surgery time would be decreased, eg. there would be no need for special diets or medication to clean the bowel. Bowel complications would be decreased and hospitalization shortened [6].

Although many (bio)materials have been developed, few groups have investigated artificial urinary conduits. In 2007 Drewa *et al.* reported successful urinary diversion in rats using an artificial, cell seeded conduit [7]. Other groups developed artificial tubes, that is epithelialized tubes prepared from minced autologous urothelium combined with fibrin or tubular degradable biopolymeric scaffolds seeded with epithelial and smooth muscle cells [8-10]. Although the outcome is promising, to our knowledge *in vivo* performance for urinary diversion remains to be demonstrated.

Using this knowledge we developed a new, large diameter construct and investigated its applicability for and function as incontinent urinary diversion in a preclinical pig model. We sought to confirm whether a tissue engineered conduit would be useful to replace bowel tissue.

Material and Methods

Collagen-Polymer Scaffolds

Preparation

Type I collagen was purified according to a previously described protocol [11]. Large tubular constructs 12 cm long and 15 mm in diameter were prepared by combining homogenized collagen (0.7% weight per volume) with and without tubularized Vypro II mesh in a cylindrical mold, followed by subsequent freezing and freeze-drying techniques [12]. Dried materials were crosslinked using carbodiimide cross-linking, [13] freeze-dried again and sterilized by 25 kGy γ irradiation (Isotron, Ede, The Netherlands).

Characterization
Scaffold biochemical composition, mechanical properties and morphology were characterized. The degree of collagen cross-linking was measured using 2,4,6-trinitrobenzene sulfonic acid [13]. Ultimate tensile strength was determined in 3 preparations on 8 x 30 mm prewet strips at an elongation speed of 2.9 mm per minute using a BioDynamic™ bioreactor. Scanning electron microscopy was used to analyze tubular scaffold ultrastructure [14].

In Vivo Study
All procedures were done according to the Institute of Laboratory Animal Research guide for laboratory animals [15]. This study was approved by the Radboud University Nijmegen Medical Centre animal ethics committee. We used 10 female Landrace pigs weighing about 50 kg each, which were housed individually with a restricted diet and free access to water.

Tubular Constructs
Urothelial cell isolation and seeding
Bladder tissue (about 4 cm^2) was harvested from the pigs under general anesthesia through a midline incision in the lower abdomen. The bladder biopsy was immersed in HBSS transport medium with Ca^{2+} and Mg^{2+}, 10 mM HEPES, 0.1% aprotinin and 1% penicillin/streptomycin. For enzymatic treatment the biopsy tissue was placed in stripping medium composed of HBSS without Ca^{2+} and Mg^{2+}, 10 mM HEPES, 0.1% aprotinin, 1% penicillin/streptomycin and 2.4 U/ml dispase II overnight at 4°C [16]. The urothelium was removed using forceps and digested in collagenase IV solution composed of HBSS with Ca^{2+} and Mg^{2+}, 10 mM HEPES and 100 U/ml collagenase IV for 20 minutes at 37°C. Urothelial cells (UCs) were suspended and collected in keratinocyte serum-free medium (Invitrogen™) with 0.5 ng/ml epidermal growth factor, 5 ng/ml bovine pituitary extract, 30 ng/ml cholera toxin, 100 U penicillin per ml and 100 µg streptomycin per ml PBS, centrifuged, resuspended and cultured on a mouse STO fibroblast feeder layer in T75 Primaria™ flasks. At 4 weeks each end of the tube was surgically closed using 2-0 polyglactin sutures. UCs were seeded by injecting 10 x 10^6 cells in the construct lumen. After cell attachment overnight the constructs were opened and cultured for 6 days. Constructs for the acellular group were kept in keratinocyte serum-free medium until implantation.

Implantation in urostomy model
The animals were divided into 2 groups. Four pigs received an acellular construct and 6 received a seeded construct (Figure 1). A median incision was made and the retroperitoneal cavity was entered via an extraperitoneal route. The right ureter was located, mobilized and transected. After gaining sufficient length (about 20 cm) the ureter was spatulated and a tension-free end-to-side anastomosis was created using 5-0 Monocryl® running sutures. The distal part of the ureter was closed. A flank

incision was made through skin, fascia and muscle on the right lateral side before the hind leg, through which the tubular construct was led. An 8Fr ureteral catheter (Vycon, Ecouen, France) was inserted and fixed using 4-0 Monocryl at the distal part of the construct. The construct was fixed to the fascia using 2-0 polyglactin sutures and to the skin using 3-0 polyglactin sutures. The laparotomy was closed using 0 polyglactin sutures for fascia, 2-0 polyglactin running sutures for subcutaneous fat and CT-1 polyglactin for skin. The catheter was removed at 21 days.

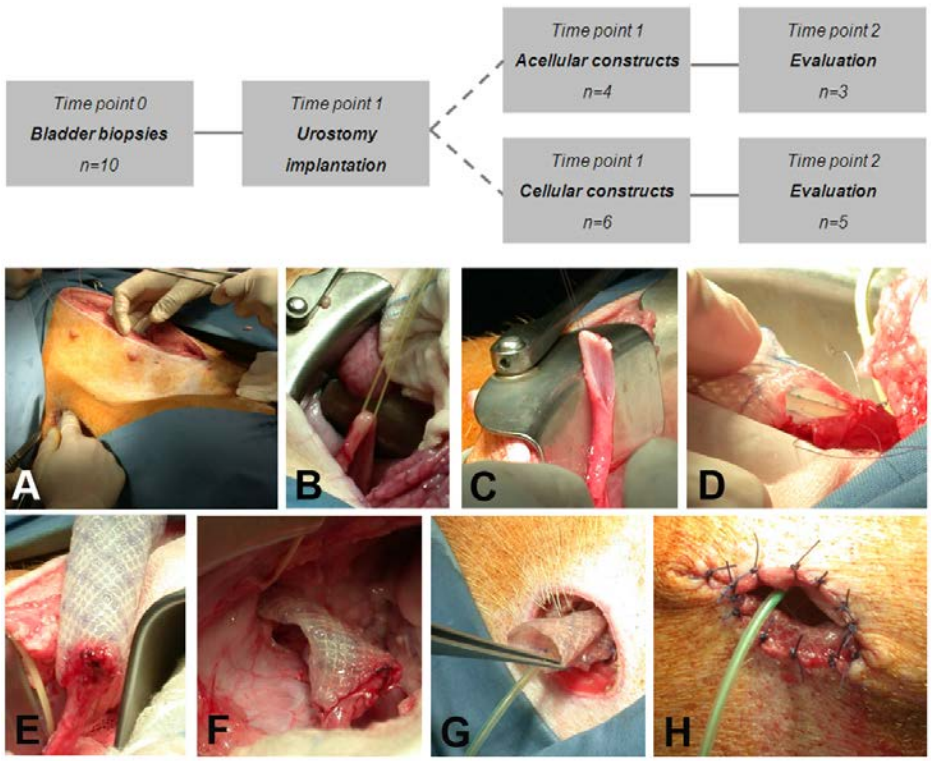

Figure 1. *Overview of urostomy construction in pig model. At 1 month (Time point 1) pigs were divided into 4 that received acellular construct and 6 that received construct seeded with autologous UCs.* ***A.*** *midline incision was made below umbilicus.* ***B.*** *right kidney and right ureter were localized.* ***C.*** *right ureter was dissected and spatulated.* ***D*** *and* ***E.*** *scaffold was anastomosed end to side to proximal ureter using running sutures.* ***F.*** *construct was passed through abdominal wall.* ***G*** *and* ***H.*** *construct was attached to fascia and skin, and straight catheter was left in ureter for 2 weeks.*

Urostomy Characterization

One month after urostomy implantation the pigs were evaluated radiologically and histologically. Loopogram was done by instilling 1:1 diluted Xenetix® 300 iodinated contrast fluid in the construct via a catheter to a pressure of 40 cm H_2O. All images were collected with a BV-25 C-arm image intensifier (Philips, Eindhoven, the Netherlands). The pigs were then sacrificed by an overdose of intravenous barbiturate. The urostomy, ureter and kidney were macroscopically inspected and fixed in 4% volume per volume formaldehyde in PBS for further histological evaluation.

Immunohistochemistry

Paraffin embedded material was cut and stained with hematoxylin and eosin [17]. Also, immunohistochemistry was done for pancytokeratin, smoothelin, vimentin, desmin and α-smooth muscle actin. Sections were deparaffinized and blocked in 3% volume per volume H_2O_2 in PBS. Antigen retrieval was performed for pancytokeratin and smoothelin using microwave treatment. All sections were incubated with 5% weight per volume horse serum for 30 minutes. Sections were incubated with primary antibodies for 1 hour. Monoclonal mouse anti-human antibodies were used, including to cytokeratins (Thermo Scientific®) (dilution 1:800) for pancytokeratin to target UCs, to desmin (1:400) to target muscle cells, to vimentin (BioGenex, San Ramon, California) (dilution 1:1,000) to target fibroblasts, to smooth muscle actin (Sigma®) (1:2,000) for α-smooth muscle actin to target smooth muscle cells and mature fibroblasts, and to smoothelin (Santa Cruz Biotechnology, Santa Cruz, California) (dilution 1:75) to target mature muscle cells.

A subsequent reaction was performed using an ABC Kit (Vector Laboratories Burlingame, California) and developed using PowerDAB (ImmunoLogic, Duiven, the Netherlands). Sections were counterstained with hematoxylin (Boom, Meppel, the Netherlands).

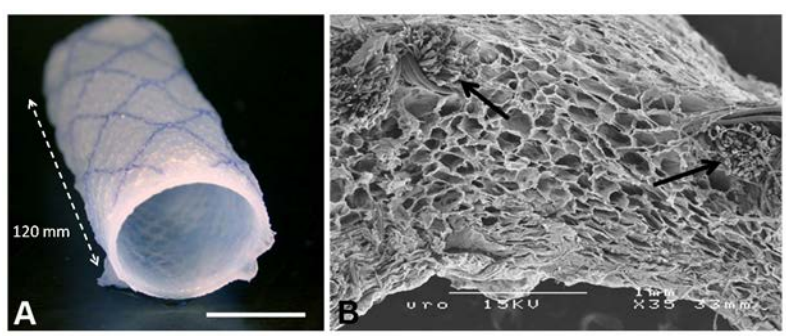

Figure 2. *Tubular construct.* ***A.*** *macroscopic overview shows 12 cm length and 15 mm diameter. Note blue Vypro II mesh.* ***B.*** *scanning electron microscopy reveals scaffold cross section with highly porous network. Note intimate contact between polymer mesh and collagen. Polymer mesh fibers (arrows) were not observed in scaffold lumen. Reduced from x35.*

Results

Scaffold Characterization

Figure 2A shows a macroscopic overview of the large collagen-polymer tubular constructs. The tubular constructs were highly porous (pore size 100 to 150 µm) and the polymer mesh was fully incorporated (Figure 2B). The collagen was successfully crosslinked, as evidenced by a 37% decrease in free amine groups. Tensile strength of the hybrid constructs was fivefold higher than a similar construct prepared from collagen only (mean ± SD 1.25 ± 0.19 vs 0.25 ± 0.04 N/mm).

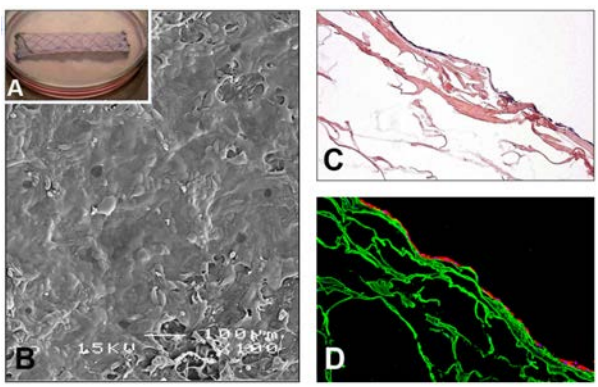

Figure 3. A. *macroscopic view of tubular scaffold in culture. B to D. microscopic views of primary UCs after seeding. Reduced from x400.* ***B.*** *scanning electron microscopy reveals almost confluent UC layer covering lumen.* ***C.*** *cross-section shows cellular lining. H&E.* ***D.*** *pancytokeratin stained positive for cellular lining inside construct, indicating that seeded cells were UCs.*

Cell Seeding and Culture

After 1 week of static culture an almost confluent layer of UCs was observed lining the construct lumen (Figure 3A-C). Immunohistochemical staining showed that seeded cells were positive for pancytokeratin (Figure 3D), confirming that only UCs were seeded on scaffolds.

In Vivo Evaluation

The survival rate after urostomy implantation was 80% (8 of 10 pigs). One pig that showed severe weight loss and lethargy was sacrificed according to regulatory guidelines (unrelated death). Stomasite stenosis developed in all animals. In 1 pig the conduit closed completely. This animal was also sacrificed before the predetermined time point (related death). To prevent stomal stenosis wound drains (Microtek Medical, Columbus, Mississippi) were positioned in the urostomy and fixed to the distal part of the stoma and to the skin. They remained in place until the end of the experiment. In all other pigs no complete obstruction was noted.

In the 8 pigs that survived followup a retroperitoneal tunnel was formed through which urine was diverted (Figure 4A). Loopograms revealed stenosis at the ureteral anastomosis in 3 pigs, of which 2 had leakage. In the remaining 5 pigs the urostomy withstood water infusion at a pressure of 40 cm H_2O. Mean conduit length and outer diameter were 10.2 ± 1.5 and 2.7 ± 0.4 cm, respectively (Figure 4C and E). The lumen of these structures had a stellate appearance (Figure 4D and 5D). In 4 cases polymer mesh was found in the lumen of the conduits and was neither degraded nor incorporated in tissue. In all pigs the right upper urinary system was affected, showing hydroureter and hydroureteronephrosis (Figure 4B).

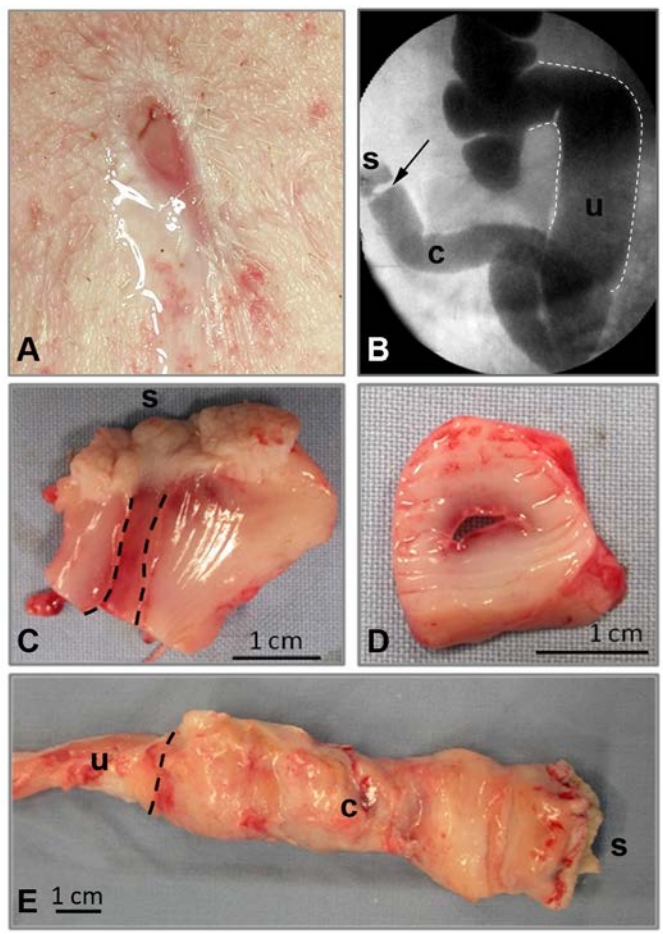

Figure 4. *Urostomy 1 month postoperatively.* ***A.*** *opening at skin level with urine.* ***B.*** *loopogram reveals stenosis (arrow) at skin level, severely dilated ureter (white dotted lines) and dilated renal pelvis.* ***C.*** *longitudinal cross section shows conduit at skin (s) side.* ***D.*** *conduit cross section.* ***E.*** *complete resected urinary conduit (c). u, ureter.*

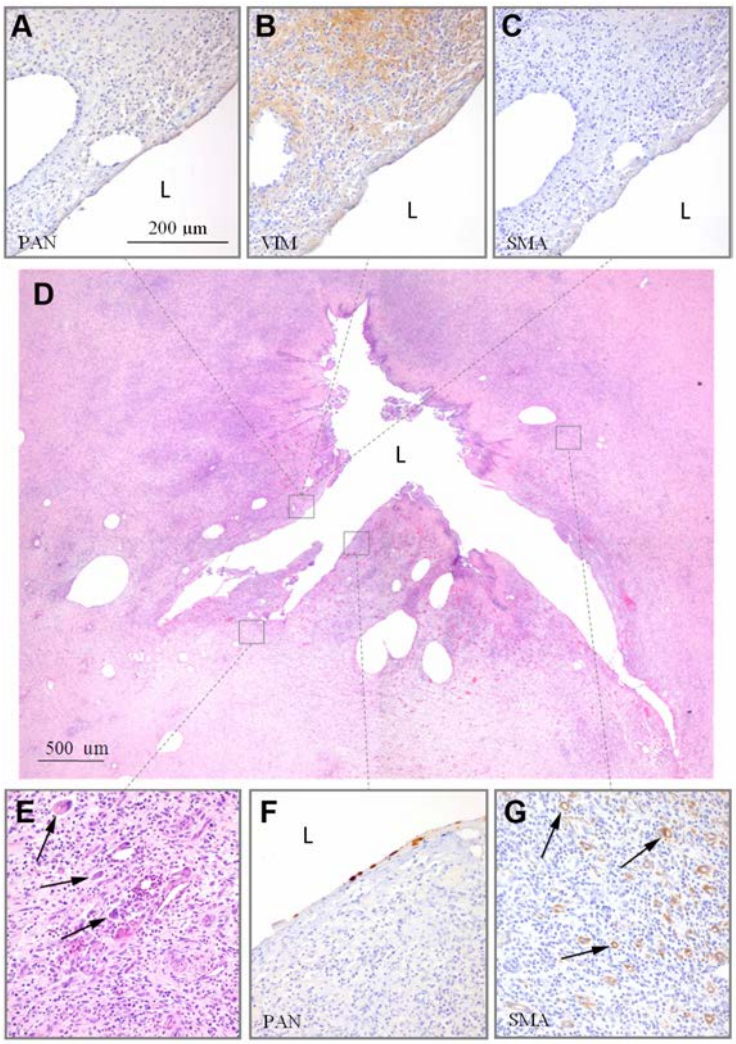

Figure 5. *Immunohistograms of urostomy tissue.* ***A*** *and* ***F.*** *urostomy inhomogeneous lining with pancytokeratin positive cells (PAN). L, conduit lumen.* ***B.*** *heterogeneously distributed vimentin-positive cells (VIM).* ***C.*** *absent smooth muscle actin positive cells (SMA).* ***D.*** *microscopic overview. Insets represent A to C and E to G.* ***E.*** *granulocytes and some multinucleated giant cells (arrows).* ***G.*** *multiple capillaries throughout entire urostomy (arrows).*

Histology

Differences between the acellular and cellular groups were not observed. Almost all collagen scaffold material was replaced by extracellular matrix (Figure 5). In 6 of the 8 pigs parts of the lumen contained pancytokeratin positive cells, confirming neo-epithelial development (Figure 5A and F). Vimentin staining was positive (Figure 5B). Most subluminal tissue was negative for desmin, smoothelin (data not shown) and smooth muscle actin except for the vasculature (Figure 5C and G), indicating fibroblasts and absent smooth muscle cells. A moderate immune response was observed, including some multinucleated giant cells (Figure 5E). Neovascularization was seen throughout the entire urostomy (Figure 5G). Regeneration at the ureteral anastomosis site was more developed with a continuous urothelial lining and smooth muscle cell ingrowth (Figure 6).

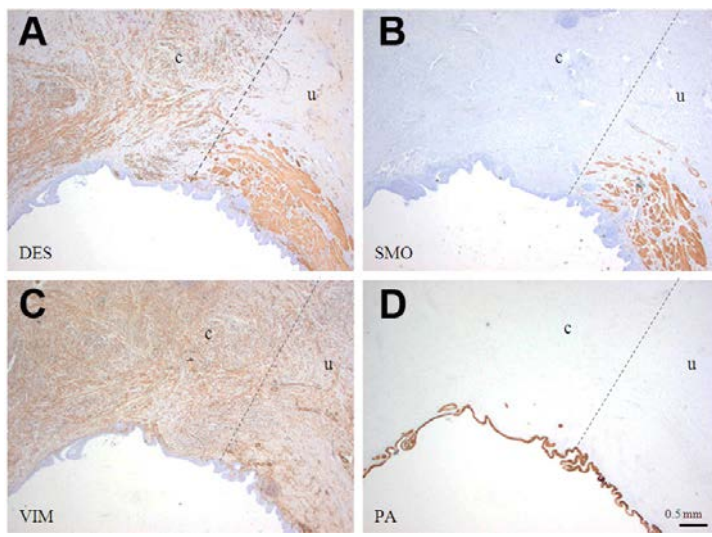

Figure 6. *Immunohistochemistry of ureteral anastomosis (dotted line).* ***A.*** *smooth muscle cell ingrowth was indicated by positive signal for desmin (DES).* ***B.*** *remodeled construct was negative for smoothelin (SMO), indicating early proliferative stage of smooth muscle development.* ***C.*** *tissue was positive for vimentin (VIM).* ***D.*** *continuous lining of ureter (u) and construct (c) with pancytokeratin positive cells (PA).*

Discussion

The most widely used solution for urinary diversion is still the ileal conduit. A large tubular construct that can function as an artificial conduit may simplify the surgical procedure and decrease GIT related complications [6]. We prepared a large, tubular collagen-polymer scaffold as long as the bowel segments currently used for urostomy [18] and tested its performance as an artificial conduit in a preclinical pig model. The constructs were implanted successfully. Watertight anastomosis was achieved between the ureter and the construct. One month after implantation collagen was resorbed and a highly vascularized retroperitoneal tunnel was formed through which urine was diverted. Although 80% of the pigs survived the experiment, necropsy examination revealed a hydronephrotic kidney and a hydroureter in all. These findings were probably caused by stenosis at the skin side of the stoma.

Several factors, including the use of 3-dimensional biomaterials, are important when developing such artificial conduits [8,19,20]. We incorporated a polymer mesh to reinforce the fragile, easily collapsible collagen, which reinforced the construct without affecting cytocompatibility. An almost confluent layer of UCs was observed on the luminal side of the scaffold within 1 week of culture. This indicates that the construct is adequate for stable attachment, proliferation and survival of other primary cells, as in a previous study [21]. At 1 month in vivo UCs were found in 6 of 8 conduits in the seeded and unseeded groups. Epithelial outgrowth and smooth muscle cell ingrowth were observed, particularly at the end of the construct with the ureteral anastomosis. This indicates that successful urostomy generation may not depend on urothelial preseeding.

Vypro II mesh was not incorporated in the tissue, indicating that it may not be biocompatible and should be replaced by a mesh produced from another material. The ideal polymer mesh for this purpose should be biocompatible, pretubularized and approved by the European Medicines Agency, and Food and Drug Administration. To our knowledge such a polymer mesh is currently unavailable. We hypothesized that the polymer mesh should support the mechanical load until sufficient tissue regeneration has occurred. Polyglactin and Dexon™ meshes were not used since they degrade within weeks and lose adequate support. However, a recent study showed that polyglactin mesh induces a less severe inflammatory reaction than Vypro II mesh when implanted retroperitoneally in pigs [22]. In view of the rapid formation of the retroperitoneal tunnel in our study it is foreseeable that rapidly resorbable polyglactin and Dexon meshes could be used. New tubular biodegradable polymer structures are necessary to meet the requirements.

Although the implanted tubular constructs functioned as a urinary conduit, necropsy examination revealed a hydroureter and a hydronephrotic kidney on the urostomy side in all pigs. Most likely this was caused by stomal stenosis, which may have been the consequence of using Vypro II mesh. This is in accordance with other studies showing that this mesh can provoke fibroblast deposition and skin contraction,

and it is poorly biocompatible [22]. Adding cells decreases fibroblast deposition, thus decreasing myofibroblast related contraction [9,23,24]. However, we noted no positive effect of applied UCs and assume that the incompatible response of the mesh decreased this effect.

On the other hand, more cell types may be needed to generate a urostomy resembling an ileal-like conduit. Since the smooth muscle compartment of the bowel segment has contractile activity, it may be important to introduce contractile smooth muscle cells in the wall of the tissue engineered construct to generate peristaltic movement and prevent hydronephrosis. In this respect careful consideration should be given to determine whether a cellular construct outweighs the disadvantages, ie increased cost and decreased clinical applicability.

Other unavoidable features that may also contribute to hydronephrosis are the quadrupedal stance, rapid growth and high intra-abdominal pressure of the animal models [10,25]. A propensity for stomal stenosis was described in another 3 studies [25-27]. All groups mentioned that the stomal complications were caused by the intrinsic skin healing properties of pigs, similar to our observation.

Despite these limitations there are not many alternatives to the pig model. An important factor is relevance to the human situation since the size and abdominal anatomy are almost analogous [28,29]. Changing the animal model may decrease stomal stenosis and obstruction but also decrease clinical relevance. Also, hydronephrotic kidneys and stenosis were seen in a rat model of urinary diversion [7].

Finally, we did not evert the construct, in contrast to the customary technique in humans, in whom the gastrointestinal segment is everted to decrease leakage between the stoma and the collection bag. Everting the ileal conduit in a porcine model prevented conduit stenosis at the skin level [3]. Accordingly everting the tubular construct may be needed to alleviate stenosis and facilitate successful stomal bag fitting.

Conclusions

When large diameter tubular constructs with defined mechanical and structural characteristics were produced and used as a urinary conduit, they generated a retroperitoneal tunnel through which urine was diverted. Since we found no significant differences between the groups, we conclude that preseeding UCs may not provide any advantage due to urothelial development. Although the current design must be improved, the tissue engineered tubular constructs may be developed into an alternative for GIT. They could represent a potential, readily available product for urinary diversion surgery.

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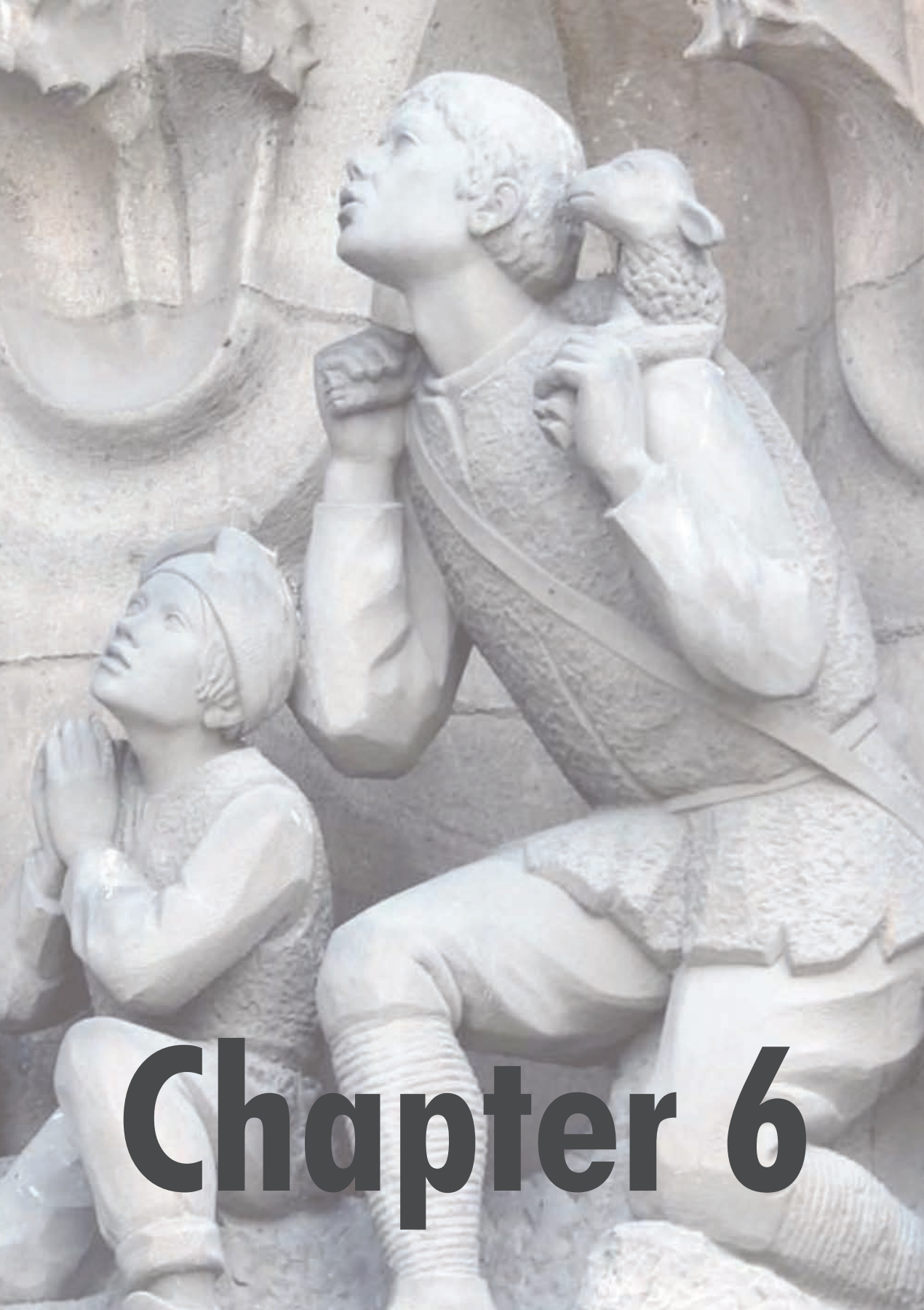

Chapter 6

Fetal Abdominal Wall Repair with a Collagen Biomatrix in an Experimental Sheep Model for Gastroschisis

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Tissue Engineering Part A 2008;14:2033-40

Abstract

We evaluated the regeneration of the abdominal wall using a dual-layer collagen biomatrix, and the protective effect on the bowel of fetal abdominal wall repair in a fetal sheep model for gastroschisis.

In 14 fetal lambs, the abdominal wall was opened at 79 days' gestation, creating a gastroschisis. In group 1, the gastroschisis was left uncovered. In group 2, the bowel was repositioned, and the defect was closed by suturing a collagen biomatrix into the abdominal wall. A cesarean section was performed at 140 days' gestation, and macroscopic and histological evaluation was performed.

In the five lambs with a gastroschisis, the eviscerated part of the bowel was coalescent, showed extensive adhesions, and was covered by fibrous peel. In group 2, the abdominal wall had closed, with a firm connection to the native abdominal wall. The biomatrix was largely degraded and replaced by connective tissue with collagen and fibroblasts, neovascularization, and scattered muscle cells. Minor or no adhesions of the bowel and no peel formation were observed.

Abdominal wall tissue replacement using a collagen biomatrix was feasible in fetal lambs, resulting in a closed abdominal wall at birth. Immediate closure of the gastroschisis strongly diminished or prevented bowel adhesions and peel formation.

Introduction

Surgical closure of congenital abdominal wall defects can be a complex problem for pediatric surgeons. An important group is patients with gastroschisis. Gastroschisis is an abdominal wall defect through which a large part of the bowel is herniated outside the abdominal cavity and is in direct contact with the amniotic fluid. The mortality in these children is approximately 10%, but intra-uterine growth retardation and premature birth are frequent, and serious complications such as sepsis, bowel dysfunction, bowel atresia, bowel necrosis, and subsequent short bowel syndrome can occur [1-4]. At birth, the bowel is often covered with an inflammatory fibrous peel; the bowel loops are matted together and can be congested or ischemic; and the bowel is thickened, inflamed, and edematous. Constriction of the bowel at the site of the abdominal wall defect and the toxic effect of the amniotic fluid cause this damage to the bowel [5,6]. The damage to the bowel seems to originate during the last trimester of pregnancy. At that time, the bowel is growing, and compression at the abdominal wall defect will occur. Furthermore, the composition of the amniotic fluid is changing because of improving kidney function and the loss of gastrointestinal waste products [7-11]. The surgical repair of these defects can be complicated using difficult primary closure because of the hypoplastic abdominal cavity and the enlarged volume of the bowel because of edema and peel formation. An increase in intra-abdominal pressure can occur after repositioning the bowel in the abdominal cavity, causing respiratory problems and compromised venous blood flow. In these cases, a gradual closure with a springloaded silo is chosen [12]. In some cases, even prosthetic materials are needed to close the abdominal wall defects or the fascia defects. These materials may also cause complications, such as wound infection, bowel fistula, erosion into abdominal viscera, lack of fixation, mesh extrusion, and adhesion formation [13]. Furthermore, patch dehiscence may occur because the material does not grow with the child [14]. Gastroschisis can be detected in early pregnancy [15], which offers the opportunity to salvage the bowel tissue using fetal therapy. Tissue-engineered constructs could be a solution for the operative closure of these defects.

The purpose of this study was to repair the full-thickness defect in the abdominal wall in fetal lambs with a surgically created gastroschisis using a molecularly defined acellular collagen biomatrix to induce regeneration of abdominal wall tissue, and to protect the bowel. We evaluated tissue regeneration in the biomatrix, the inflammatory response, the protective effect of abdominal wall closure with the biomatrix on bowel tissue, adhesions of the biomatrix to underlying tissue, and the presence of hernias.

Material and Methods

The Ethical Committee on Animal Research of the Radboud University Nijmegen Medical Centre approved this study under protocol number RUDEC 2003-96.

Preparation of collagen biomatrices

The molecularly defined, biocompatible, biodegradable dual-layer collagen biomatrices were made from insoluble highly purified type I collagen from bovine Achilles tendon [16]. The biomatrix consisted of a porous layer and a dense film layer. A 0.8% (w/v) type I collagen suspension was shaken overnight in 0.25M acetic acid at 4°C and homogenized on ice using a Potter-Elvehjem homogeniser. Air bubbles were removed by centrifuging at 250 g for 10 min at 4°C. The suspension was slowly poured into a plastic mold (4mL per Ø 32 mm), and air-dried for 3 days at 22°C to prepare a flat film layer. These films were incubated for 60 min in 4mL 0.25M acetic acid. Acetic acid was then removed, and a suspension of 4mL 0.8% type I collagen in 0.25M acetic acid was poured on top of the films, quickly frozen at -80°C, and lyophilized in a Zirbus lyophiliser (Bad Grund, Germany). Scaffolds were cross-linked using 33mM 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide and 6mM N-hydroxysuccinimide in 50mM 2-morpholinoethane sulphonic acid pH 5.0 containing 40% ethanol (5mL per Ø 32mm) for 4 h at 22°C. Scaffolds were then washed with 0.1M disodium hydrogen orthophosphate, 1M sodium chloride (NaCl), 2M NaCl, and MilliQ water, frozen again in ethanol/carbon dioxide, and lyophilized [17,18]. Biomatrix morphology was assessed using scanning electron microscopy (Figure 1) [17,18]. The porous layer had interconnective pores, and the mean average pore size of the top side of the porous layer was 106 ± 22 mm and

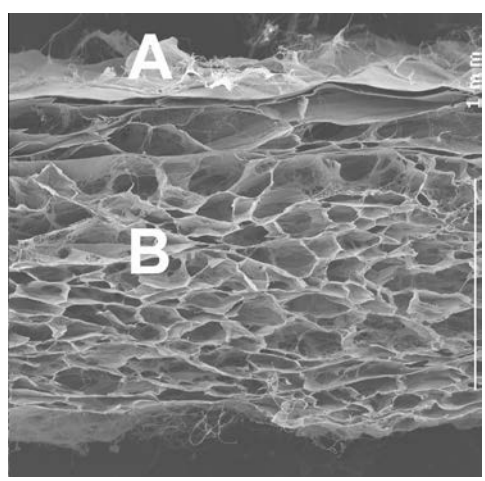

Figure 1. *Dual-layer collagen biomatrix (scanning electron microscopy).* ***A.*** *Film layer of biomatrix;* ***B.*** *porous layer of biomatrix.*

of the cross-section was 123 ± 34 mm (average of 100 pores of three individually prepared biomatrices). The porous layer and film layer had an average diameter of 1.5 mm and 2 to 3 µm respectively. Crosslinking was verified according to its amine group content, and 48% of the amine groups were used in the crosslinking process [17]. Before implantation, the matrices were washed in 70% (v/v) ethanol and sterile phosphate buffered saline.

Surgical procedures

Fourteen pregnant sheep (Dutch Texels breed) were operated on at 79 days' gestation (full term 140-147 days). An intravenous injection of 30 mg/kg pentobarbital and 1mL atropine was used for anesthesia and, following endotracheal intubation, was maintained with 2% isoflurane and oxygen/air ventilation at a respiration rate of 16 breaths per minute. The uterus was exteriorized through a midline abdominal incision. A hysterotomy was performed, and the lower part of the fetal body was exposed. In case of twin or triplet pregnancy, only one fetus was operated on to avoid additional risk of complications.

The animals were divided into two groups. In group 1, consisting of five fetuses (two male, three female), a gastroschisis was surgically created. An incision of 2.5 cm was made in the left lower quadrant of the abdominal wall of the fetuses, through skin and fascia, resulting in a full-thickness abdominal-wall defect of approximately 2.5 x 2 cm. The defect was left-sided to avoid injury to the liver by surgical manipulation. Subsequently, the bowel was exposed and gently extruded from the abdominal cavity (Figure 2A). The lesion was left uncovered, leaving the bowel exposed to the amniotic fluid.

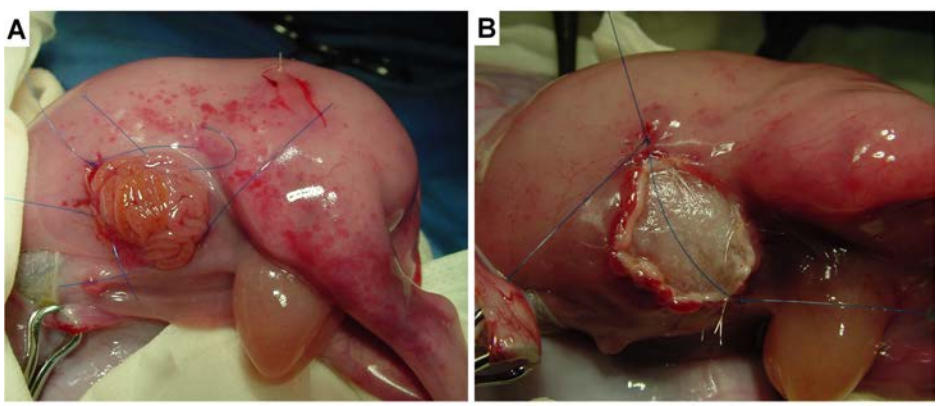

Figure 2. *Fetal lambs operated on at 79 days' gestation.* ***A.*** *Surgically created gastroschisis.* ***B.*** *Surgically created abdominal wall defect closed with collagen biomatrix.*

In group 2, consisting of nine fetuses (seven male, two female), a gastroschisis was created as in group 1. Subsequently, the bowel was gently manipulated back into the abdominal cavity, and the dual-layer collagen biomatrix, measuring approximately 2.5 x 2 cm was placed into this defect (film layer at the luminal site). The biomatrix was sutured in the abdominal wall using 6-0 poliglecaprone (Monocryl, Ethicon, Inc., Sommerville, NJ) interrupted sutures (Figure 2B). Four 6-0 polypropylene (Prolene, Ethicon, Inc.) marking sutures were placed around the biomatrix for future reference. After the surgical procedure, the fetus was returned to the uterus and amniotic fluid volume was restored using warm sterile saline together with amoxicillin 250 mg. The uterus was closed in two layers using a 2-0 polyglactin (Vicryl, Ethicon, Inc.) running suture. Sodium-penicillin (1,000,000 IU) was instilled into the intra-abdominal space, and the maternal laparotomy was closed in two layers using 1 polyglactin interrupted sutures. Depomycin (20mg/kg, subcutaneous) was initiated preoperatively and maintained postoperatively for 3 days.
At 140 days' gestation, 61 days after surgery, the lambs were delivered by caesarean section under local anaesthesia with 20 to 30 mL Lidocaine 2%, administered subcutaneously and intramuscularly.

Neonatal outcome and evaluation

After birth, the macroscopic appearance of the bowel was observed and photographed in group 1, and the size of the defect was measured. The replaced part of the abdominal wall at the place of the incorporated biomatrix in group 2 was macroscopically observed, palpated, photographed, and measured. Subsequently, the lambs were sacrificed using medetomidine (0.5 mg intramuscular) and pentobarbital (60 mg/kg intracardial). Afterward, the abdominal wall of the lambs from group 1 was opened, and the bowel was taken out from pylorus to rectum, with a small part of the adhering abdominal wall. The intra- and extra-abdominal part of the bowel was evaluated for adhesions and fibrous peel. The abdominal wall of the lambs from group 2 was opened with broad margins around the site of the former biomatrix. Intra-abdominal adhesions were observed and photographed. The abdominal wall and the bowel, from pylorus to rectum, were taken out.

Histological staining

From the lambs of group 1, tissue samples were taken from the bowel situated outside of the abdominal cavity and from bowel inside the abdominal cavity. In group 2, samples from the replacing tissue at the site of the implanted collagen biomatrix and tissue samples of the bowel lying underneath the site of the implanted collagen biomatrix were taken. Tissue samples of normal bowel of five lambs that had undergone a fetal operation in another study served as controls.
The tissue samples were fixated in 4% buffered formalin and paraffin embedded for routine histological processing. Sections (4 mm) were cut and stained with hematoxylin and eosin and Masson's trichrome. The intestinal tissue of the lambs

was examined for changes in the mucosal, submucosal, muscle, and serosal layers, and peel and adhesion formation. Two bowel samples were used from each fetus to assess the thickness of the intestinal muscularis and the serosal peel layer in three random fields with an ocular micrometer at magnification x100. The specimens, which included the site of the implanted collagen biomatrix in the abdominal wall, were examined for evidence of epithelialization, smooth muscle cell growth, neovascularization, and degradation of the biomatrix. In addition, the thickness of the replacing tissue was measured using the ocular micrometer. Immunohistochemical staining was performed using desmin for staining of muscle cells and S-100 staining to visualize nerve fibres.

Data analysis

Data analysis of the bowel measurements was performed using SPSS 12.0 for Windows (SPSS, Inc., Chicago, IL) and expressed as means ± standard deviations. Statistical analysis was performed using one-way analysis of variance. $P<0.05$ was considered statistically significant.

Results

Eleven of the 14 operated fetuses (79%) were born alive. Two fetal deaths occurred in group 2 without a clear reason; the lambs were found macerated at the caesarean section. One ewe was euthanized because of an infection in the fascia of the abdominal wall, resulting in uncorrectable fascia dehiscence. No further maternal deaths occurred.

The five control lambs showed no macroscopic or histological bowel changes. At histological examination, the serosa measured 0.02 ± 0.01 mm and the intestinal muscularis 0.08 ± 0.03 mm.

After birth, four lambs of group 1 showed eviscerated bowel covered with a fibrous peel; in two lambs, a thick layer was present, and in two lambs a clearly thinner layer of fibrous peel was visible (Figure 3A). In one lamb, the bowel had spontaneously repositioned into the abdominal cavity, and the abdominal wall had closed with a small scar. The bowel of this lamb was enveloped in a sac, with the same appearance as the fibrous peel. The eviscerated bowel package of the other four lambs measured approximately 7 x 5 x 5 cm in size, and the size of the abdominal wall defect was 5 cm. The eviscerated part of the bowel was coalescent and showed extensive, inseparable adhesions. The intra-abdominal part of the bowel also showed adhesions, although of a much lesser extent than the eviscerated bowel. No atresia or other bowel abnormalities were seen.

Histological examination of the intestines of the gastroschisis lambs (group 1) showed that the serosa of the four lambs with the eviscerated bowel was covered

with fibrous peel. In two lambs, the fibrous peel was thick (mean diameter 1.37 ± 0.36 mm and 2.64 ± 0.91 mm). The peel consisted of deposited fibrin and degenerated granulocytes, granulation tissue with chronic inflammation (focal foreign body giant cells around hair remnants, plasma cells, lymphocytes, and histiocytes), fibrosis, and focal hemosiderin pigment. In two lambs, the fibrous peel was thinner (0.75 ± 0.23 mm) and without inflammation. In these lambs, a pseudo-epithelial mesenchymal layer of cells, which seemed to protect the bowel tissue against the amniotic fluid, covered the peel (Figure 3B). No edema, venous dilatation, lymphatic dilatation, or signs of ischemia were seen in the bowel tissue. The mucosa appeared normal, with normal villi, and the submucosa was normal, without collagen deposits. The intestinal muscularis showed some thickening (0.14 ± 0.05 mm) with collagen deposition; normal ganglion cells were seen. The bowel of the lamb with the spontaneously closed defect showed no abnormalities, with a normal serosal layer (mean diameter 0.02 mm).

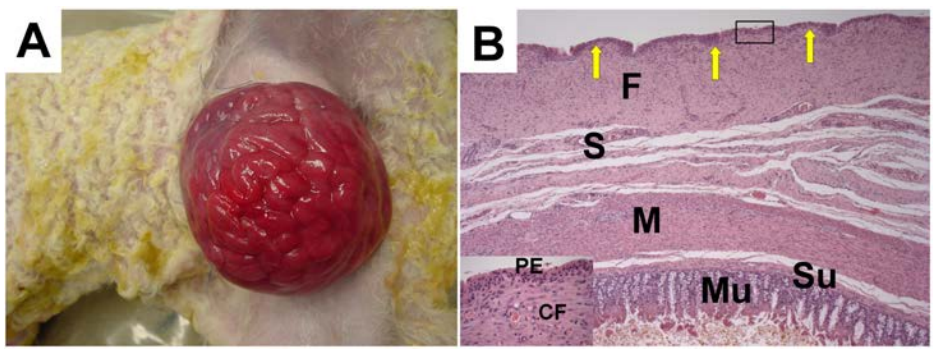

Figure 3. A. *Macroscopic aspect of the surgically created gastroschisis (group 1), after birth, showing coalescent bowel loops covered with fibrous peel.* ***B.*** *Histological picture of the bowel wall of a lamb from group 1, after birth, showing the fibrous peel. Arrows = pseudoepithelial layer of mesenchymal cells; F, fibrous peel; S, subserosal layer; M, intestinal muscularis; Su, submucosa; Mu, mucosa (hematolyxlin and eosin (H&E) staining, original magnification x50). (Magnification of rectangle in inset). Inset: PE, pseudoepithelial layer of mesenchymal cell; CF, collagen and fibroblasts (H&E staining; x40).*

The six surviving lambs of group 2 showed a closed abdominal wall; the replacing tissue was visible between the marking sutures in the skin after shaving, measuring approximately 3.2 ± 0.8 cm in diameter (Figure 4A). The replacing tissue was of strong consistency; in one lamb, a small herniation in the abdominal wall of 1.5 cm was palpable under the skin, and in the other lambs, no herniations were palpable. In the center, a hyperkeratotic area was seen in five of six lambs, and the surrounding tissue formed a ridged configuration. Underneath this regenerated tissue, the bowel had a normal appearance, no adhesions between bowel loops were visible, and only some minor adhesions between the bowel and the abdominal wall existed (Figure 4B).

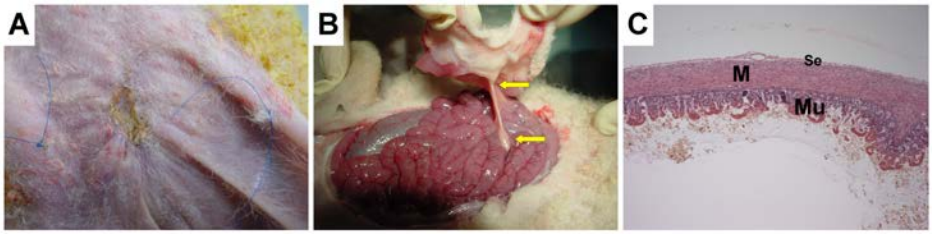

Figure 4. A. *Abdominal wall defect closed using a collagen biomatrix (group 2), macroscopic aspect after birth, showing a closed abdominal wall with regenerated skin tissue.* ***B.*** *Minor adhesions visible (arrows) in a lamb from group 2. (**C**) Histological picture of the bowel of a lamb from group 2, showing no abnormalities. Se, serosal layer; M, intestinal muscularis; Mu, mucosa (hematolyxlin and eosin staining; original magnification x50).*

The bowel tissue in these lambs appeared normal on histological examination. No peel formation was found; the serosal layer (0.02 ± 0.01 mm) and the intestinal muscularis (0.08 ± 0.03 mm) were of normal thickness and significantly thinner (both $p<0.001$) than in gastroschisis lambs. The submucosa and mucosa appeared normal (Figure 4C).

Histological examination of the abdominal wall showed tissue replacement throughout the entire biomatrix in all lambs. Connective tissue, with collagen and fibroblasts, was largely replacing the biomatrix. There was a firm connection with the adjacent skin, subcutaneous tissue, and muscle of the native abdominal wall. The replacing tissue was thinner than the native abdominal wall, with the native abdominal wall measuring 6.2 ± 1.4 cm and the replacing tissue 3.6 ± 1.4 cm (Figure 5A,B,D). On the outside, the entire replacing tissue was covered with skin tissue in all lambs. The skin tissue was more mature at the edges of the newly formed tissue, with epithelialization and adnexal differentiation, including sebaceous glands and hair follicles, than at the center of the tissue (Figure 5A). Tissue replacement occurred from the borders of the regenerated tissue. Hyperkeratinization was visible at the center of this tissue in five of six lambs (Figure 5B). Good neovascularization was seen throughout the entire replacing tissue, at the borders as well as the center of this tissue (Figure 5A-C). The angiogenesis seemed to originate from the peritoneum and the edges of the native abdominal wall. Only minor inflammatory reaction was seen, without a foreign body reaction. The collagen biomatrix was largely degraded, except for the less-porous film layer, which was still visible at the inside of the abdominal wall (Figure 5A-C). A small number of scattered muscle cells were visible in the replacement tissue, surrounded by myofibroblasts, but no muscle bundles were seen (Figure 5D,E). Only few nerve fibers could be found in this tissue (Figure 5A).

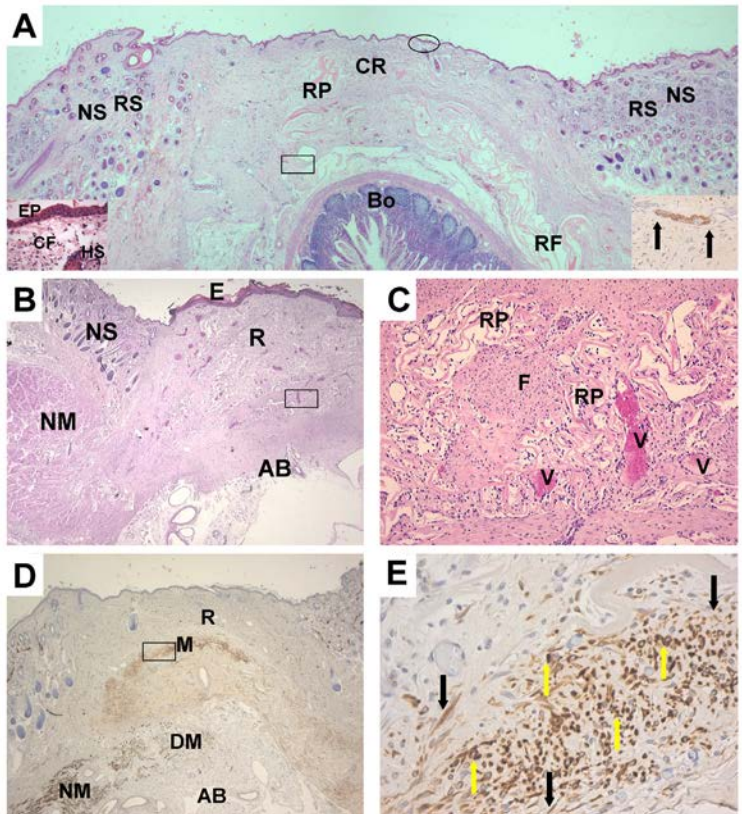

Figure 5. *Histological images of the replacing tissue at the side of the abdominal wall defect closed using a collagen biomatrix (group 2).* ***A.*** *Overview of replacement tissue showing that regenerated skin and connective tissue replaced the biomatrix. The regenerated skin tissue (RS) (=between RS) at the border was more mature, with hair follicles and sebaceous glands, than at the center (CR). The replacement tissue was thinner than the native abdominal wall. Remnants of the film layer (RF) and porous layer (RP) were visible. NS, native skin; Bo, bowel. (Hematolyxlin and eosin (H&E) staining; original magnification x12.5). Inset on the left: magnification of the ellipse at the overview, showing epidermis (EP), collagen and fibroblasts (CF), and hair follicle and sebaceous gland (HS) (H&E staining; x40). Inset on the right: nerve fibre in regenerated tissue (arrows) (S-100 staining; x200), located at the rectangle in overview.* ***B.*** *Overview of regenerated tissue, showing the border between native skin (NS) and native muscle (NM) and the replacement tissue (R). The skin is covered with a hyperplastic epidermis with hyperkeratinization (E). AB, abdominal site. (H&E staining; original magnification x25).* ***C.*** *Connective tissue formation, with fibroblasts and collagen (F) and mature vessels (V). RP, remnant of porous layer biomatrix. (Magnification of rectangle in Fig. 5B). (H&E staining; x100).* ***D.*** *Replacement tissue (R) with scarce smooth muscle cell formation (M). The native muscle (NM) was partially degenerated (DM). AB, abdominal site. (Desmin staining; x12.5).* ***E.*** *Magnification of smooth muscle cells; yellow arrows = rounded striated muscle cells; black arrows = spindle-shaped myofibroblasts. (Magnification of rectangle in Fig. 5D). (Desmin staining; x200).*

Discussion

Fetal therapy has been applied for several congenital anomalies in experimental settings. This was first performed in life-threatening anomalies, but nowadays it is also used for the treatment of non-fatal anomalies, as in gastroschisis [2]. The survival rate in gastroschisis patients is commonly greater than 90%, but various problems may arise after birth, and some report a postnatal complication rate of 79% [4]. The damage to the bowel is assumed to occur during late pregnancy, due to constriction of the bowel at the abdominal wall defect and exposure to the amniotic fluid [5]. Early detection of gastroschisis during pregnancy is possible using routine ultrasound screening and offers the opportunity for early treatment during the fetal period, to protect the bowel tissue against further secondary injury. Till *et al.* successfully repaired the abdominal wall of rabbit fetuses with a surgically created gastroschisis using operative closure, but the follow-up period was negligible [19]. Tissue-engineering techniques have been used to close abdominal wall defects in adult animals. In these studies, full- or partial-thickness abdominal wall defects were surgically created, and scaffolds of extracellular matrix were used to close these defects, resulting in firm connective tissue formation, degradation of the scaffold, and some reported regeneration of skeletal muscle [14,20-25]. Acellular scaffolds have also been used experimentally in small numbers of humans with large abdominal wall defects that were inappropriate for primary closure [26,27]. In previous work, we used a collagen biomatrix to cover a surgically created neural tube defect in fetal lambs [28,29].

In the present study, we used the fetal lamb model for surgical creation of gastroschisis. The lambs in which the bowel was left eviscerated out of the abdomen showed macroscopic and histological similarity with gastroschisis in humans. Extensive adhesions and peel formation were seen in these lambs.

In one lamb, the bowel had repositioned in the abdomen, and the abdominal wall had closed. Spontaneous intrauterine closure of the defect in humans has been reported, accompanied by intestinal involution resulting in intestinal atresia [15,30]. In our animal model, the defect in the abdominal wall was larger than the defects in humans, which may explain the spontaneous repositioning of the bowel in one lamb, and the absence of signs of bowel constriction at time of birth.

In the other group, the full-thickness abdominal wall defect was immediately repaired using a collagen biomatrix, and the regeneration of abdominal wall tissue and possible changes of the bowel were studied. We used a molecularly defined, biocompatible, biodegradable dual-layer biomatrix of highly purified type I collagen, which is a modification of the biomatrix previously used for fetal closure of spina bifida, to repair the abdominal wall [28,29,31]. The modification consisted of adding an additional thin layer of collagen with less porosity and higher strength capabilities, to increase the total tensile strength of the biomatrix. In all lambs, the defect in the abdominal wall had closed after birth. Histological examination

showed that the porous layer of the biomatrix was largely degraded, but the film layer resided. Connective tissue and skin formation replaced the porous layer of the collagen biomatrix. Good neovascularization occurred throughout the entire replacement tissue. No inflammatory response to the biomatrix was visible. It is likely that this was due to the use of highly purified collagen, instead of decellularized tissue that is often used in other animal studies. The tissue appeared to be firm and was well incorporated into the native abdominal wall tissue. In one lamb, a small herniation was palpable; in the other five lambs, no herniations occurred. Only a small group of muscle cells was seen in the repaired area, surrounded by spindle-shaped myofibroblasts. Some groups have reported varying degrees of muscle regeneration, [21-25] but the follow-up period of 61 days in our study could be too short; the tissue would probably have developed further during the postnatal period if the lambs had not been sacrificed. The bowel appeared normal on macroscopic and histological examination. Only minor adhesions between the bowel and the abdominal wall occurred with this collagen scaffold, without further complications, and no peel formation or inflammatory reaction was seen in the bowel. Immediate closure of the abdominal wall defect prevented these alterations.

In human studies in which a collagen biomatrix was used to close abdominal wall defects, the major disadvantage was the low tensile strength of the collagen biomatrix, especially when degradation of the collagen occurred [13]. By modifying the biomatrix using chemical cross-linking of the collagen, the degradability can be adjusted to make it more appropriate for abdominal wall closure, and the strength is greater [16-18]. The addition of the film layer with less porosity further improves the strength and maintains this strength for a longer period because of its lower degradability. Furthermore, improved regeneration of the muscle and fascia would also provide extra strength to the newly formed tissue. A possible strategy of improving the regeneration could be the incorporation of growth factors into the biomatrix to enhance neovascularization and ingrowth of (muscle) cells. The incorporation of autologous muscle cells also seems to improve cell infiltration and mechanical performance [22-25,32,33]. However, this technique may be inappropriate for fetal operations, because an additional operation for muscle biopsies will then be needed, increasing the risk of complications.

The use of biomaterials can be a promising tool for patients with an abdominal wall defect to regenerate the abdominal wall or cover the eviscerated bowel. Fetal therapy may have its advantages in these patients; early coverage of the bowel could prevent inflammatory changes and adhesion formation and might salvage the bowel function. Furthermore, the defect is smaller at this point, adequate neovascularization may be obtained throughout the entire biomatrix, and the incongruence of volumes between the abdominal cavity and the bowel is smaller than in neonates. Fetal wound healing has a strong potency and can even result in scarless wound healing [34]. By repairing these defects at the fetal stage, the advances of the regenerative capacity of the fetus are used. However, in our model,

the regeneration process took place during gestational weeks 11 through 20, which is largely during the third trimester. Scarless wound healing and the regenerative capacity of the fetus decrease when the fetus is nearing full term, and near term it will be comparable with postnatal wound healing [34].
Currently, the major disadvantage of fetal surgery is the risk of complications leading to premature delivery [35,36]. However, with improvements in the techniques for fetal access, it can become a promising tool for patients with this congenital anomaly in the near future.

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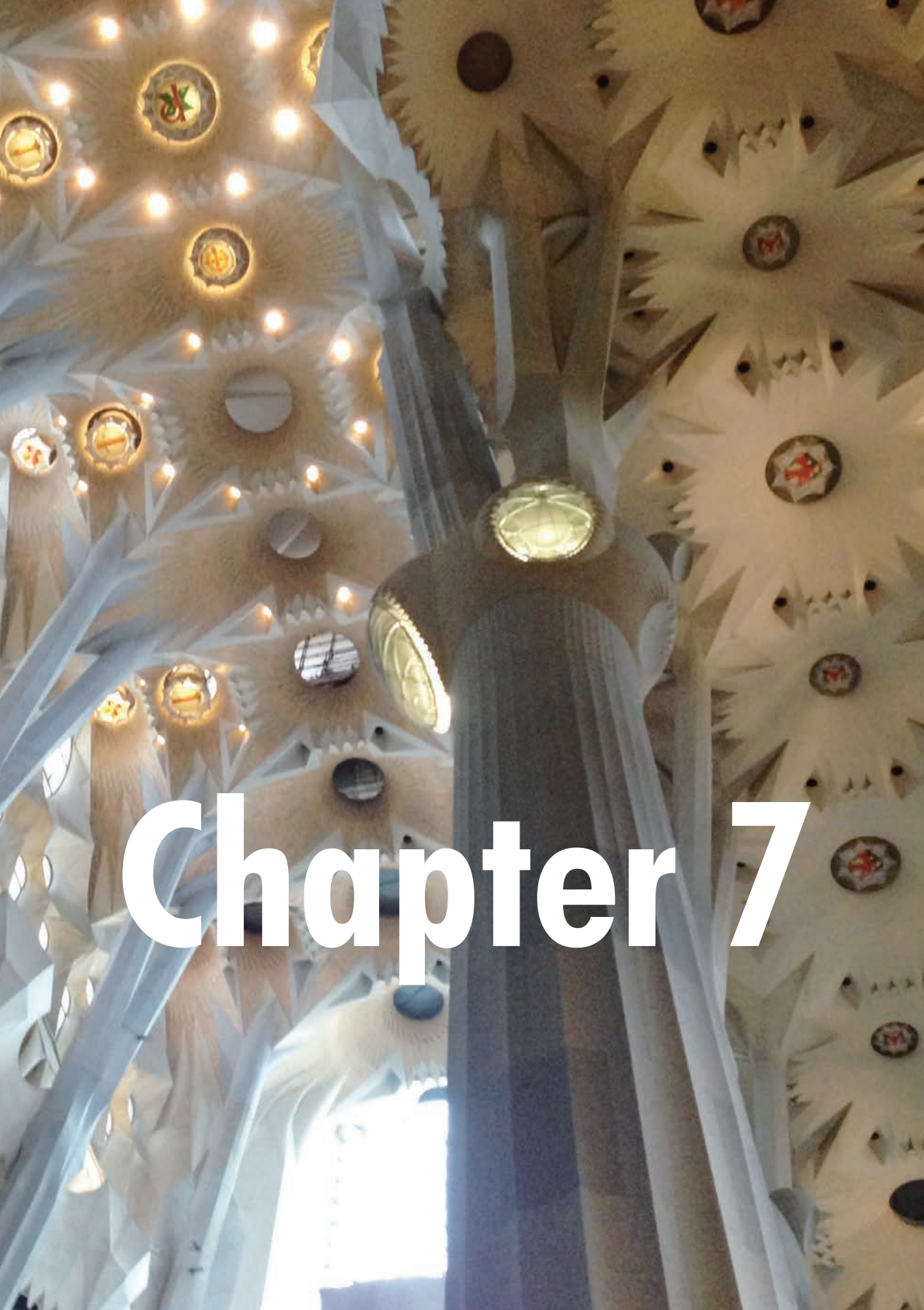

Chapter 7

Prenatal Coverage of Experimental Gastroschisis with a Collagen Scaffold to protect the Bowel

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Journal of Pediatric Surgery 2013;48:516-24

Abstract

Background/Purpose: In fetuses with gastroschisis, toxic products in the amniotic fluid and constriction at the defect of the abdominal wall are considered causative of damage to the eviscerated bowel. The aim of this study was to cover the eviscerated bowel in gastroschisis with a collagen scaffold to protect the bowel and induce cell growth into the scaffold, which could lead to skin or abdominal wall formation replacing the scaffold.

Methods: In 12 fetal lambs gastroschisis was surgically created at 79 days gestation. A dual-layer type I collagen scaffold was sutured into the skin of the abdominal wall around the defect covering the eviscerated bowel. Lambs were examined after caesarean section at 140 days' gestation.

Results: Survival was 67%. In 7 of 8 surviving lambs the bowel was found to be covered after birth. One scaffold had ruptured. The bowel was found repositioned in the abdominal cavity in 5 lambs. In 2 lambs it was still partially outside. Only minor adherence of bowel loops and no fibrous peel formation were seen. Connective tissue and skin tissue replaced the scaffold.

Conclusions: Prenatal coverage of the bowel in experimental gastroschisis with a collagen scaffold is feasible in fetal lambs, significantly diminished damage to the bowel wall, and skin and connective tissue replaced the scaffold. This technique may be promising in the care of fetuses with this congenital anomaly.

Introduction

Gastroschisis is an abdominal wall defect, resulting in herniation of a large part of the bowel outside the abdominal cavity, where it is in direct contact with the amniotic fluid. Mortality in neonates with gastroschisis is reported to be 4%-12.5%. Intra-uterine growth retardation and premature birth are frequently noted, and serious complications like sepsis, bowel dysfunction, bowel atresia, bowel necrosis and subsequent short bowel syndrome may occur. Intestinal motility and absorption are decreased and postnatal feeding can be problematic [1-4]. At birth, the bowel is often covered with an inflammatory fibrous peel, and bowel loops are matted together, can be congested or ischemic, and are thickened, inflamed and edematous. This damage to the eviscerated bowel may be the result of constriction at the site of the abdominal wall defect and/or the toxic effect of the amniotic fluid [5,6]. Damage to the bowel seems to occur during the last trimester of pregnancy, when the bowel is growing, causing compression at the site of the abdominal wall defect. Additionally, the composition of amniotic fluid is changing due to the improving kidney function and the release of gastrointestinal waste products into the amniotic fluid [7-11]. Primary closure of the abdominal wall can be problematic because the abdominal cavity is relatively hypoplastic and the bowel volume is enlarged due to edema and fibrous peel formation (visceral-abdominal disproportion). Repositioning the bowel into the abdominal cavity will increase the intra-abdominal pressure, and may result in respiratory problems, compromised venous blood flow and abdominal compartment syndrome. In these cases a gradual abdominal wall closure with a spring-loaded silo is often employed [12]. Occasionally prosthetic materials are needed to close the abdominal wall or the fascia defects. These materials may also cause complications, including wound infection, bowel fistula, erosion into abdominal viscera, lack of fixation, mesh extrusion and extreme adhesion formation [13]. Furthermore, patch dehiscence may occur because the material does not grow with the child [14].

Tissue-engineered constructs can be useful for the operative closure of these defects, either prenatal or in the neonatal period. A carrier material of extracellular matrix is provided to the tissue. Cell ingrowth into this scaffold, replacing and degrading the scaffold, will result in newly formed or regenerated autologous tissue. We developed a molecularly defined, biocompatible, acellular, single or dual-layer porous scaffold from type I collagen derived from bovine Achilles-tendon [15-17]. In previous animal research this collagen scaffold was used to augment the bladder [18], repair fetal skin defects [19], and close a full-thickness abdominal wall defect [20].

Routine ultrasound screening during pregnancy allows early detection of gastroschisis, and offers the potential option for early treatment during the fetal period, to protect the bowel from secondary injury [21].

The purpose of this study was to evaluate the protective effect of intra-uterine coverage of the eviscerated bowel tissue in fetal lambs in which gastroschisis was surgically created, aiming at preventing fibrous peel and adhesion formation. For this purpose we used the previously used dual-layer collagen scaffold. Findings were compared to a historical control group of lambs, from a previous study, in which the eviscerated bowel was left uncovered [20]. Additionally, we aimed at inducing cell ingrowth into the scaffold, ultimately resulting in skin or abdominal wall tissue replacing the scaffold and covering the vulnerable bowel loops.

Materials and methods

The study was conducted under the supervision of veterinarians according to National Institutes of Health (NIH) guidelines for the care and use of laboratory animals (NIH publication 85-23 Rev. 1985). The study was approved by the Ethical Committee on Animal Research of the Radboud University Nijmegen Medical Centre.

Preparation of collagen scaffolds

Molecularly-defined, biocompatible and biodegradable dual-layer collagen scaffolds were made from insoluble, highly purified, type I collagen from bovine Achilles tendon [15-17]. The scaffold consisted of a porous layer and a dense film layer. The preparation and characterization of this scaffold were previously described [20]. Before implantation the scaffolds were disinfected in 70% (v/v) ethanol and washed with sterile phosphate-buffered saline (PBS).

Surgical procedures

Twelve pregnant sheep (Dutch Texel breed) were operated at 79 days' gestation (full term 140-147 days). Anesthesia was induced by pentobarbital (30 mg/kg intravenous, AST Pharma, Oudewater, the Netherlands) and atropine sulphate (1 ml intravenous, Pharmachemie BV, Haarlem, the Netherlands) and, following endotracheal intubation, maintained with 2% isoflurane (Nicholas Piramal, London, UK) and O_2/air ventilation at a respiration rate of 16 per min. Heart rate, temperature, oxygen saturation and carbon dioxide concentration of the expired air were monitored. The abdomen was shaved, cleaned and aseptically prepared. A lower midline abdominal incision was made. One horn of the uterus was exteriorized, and wrapped in gauze soaked in warmed PBS. A hysterotomy was performed and the lower part of the fetal body was exposed. In case of twin or triplet pregnancy only one fetus was operated to avoid additional risk of complications.

In 12 fetuses (5 male, 7 female), gastroschisis was surgically created as previously described [20]. An incision of 2.5 cm through skin, fascia, muscle and peritoneum was made in the left lower quadrant of the abdominal wall, resulting in a full-thickness

abdominal wall defect of approximately 2.5 x 2 cm. The defect was created on the left side to avoid injury to the liver. The bowel was exposed and gently extruded from the abdominal cavity, to create a gastroschisis-like lesion. Subsequently the skin around the defect was ovally incised at approximately 3 mm from the edges of the defect. The skin edges of this oval wound were carefully detached from the underlying fascia (Figure 1A). The dual-layer collagen scaffold, measuring approximately 3 x 3.5 cm was placed into this oval skin defect (film layer at the intestinal site), covering the eviscerated bowel. The scaffold was sutured to the skin and the abdominal wall with 6-0 polyglecaprone (Monocryl®, Ethicon Inc.; Sommerville, NJ, USA) running sutures (Figure 1B,C). Four 6-0 polypropylene (Prolene®, Ethicon Inc.; Sommerville, NJ, USA) marking sutures were placed around the scaffold for future reference.

After the surgical procedure the fetus was returned into the uterus and amniotic fluid volume was restored with warmed sterile PBS together with Amoxicillin (250 mg, Centrafarm Services B.V., Etten-Leur, the Netherlands). The uterus was closed in two layers with a 2-0 polyglactin (Vicryl®, Ethicon Inc.; Sommerville, NJ, USA) running suture. Sodium-penicillin (1,000,000 IU, Astellas Pharma, Leiderdorp, the Netherlands) was instilled into the intra-abdominal space and the maternal laparotomy was closed in two layers using 1 polyglactin interrupted suture. Depomycin (20,000 IU penicillin ml^{-1}, streptomycin ml^{-1}, 20 mg/kg, subcutaneous, Intervet, Boxmeer, the Netherlands) was initiated preoperatively and maintained postoperatively for three days.

At 140 days' gestation, 61 days after fetal surgery, the lambs were delivered by caesarean section under local anaesthesia with Lidocaine 2% (20-30ml, subcutaneous and intramuscular, Fresenius Kabi, Den Bosch, the Netherlands).

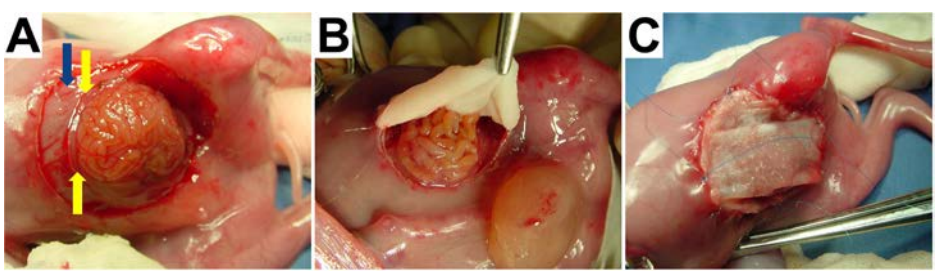

Figure 1. *Surgery on fetal lambs at 79 days gestation.* ***A.*** *Surgically created gastroschisis; blue arrow: oval incision of skin around defect; yellow arrows: skin between incision and defect.* ***B.*** *Gastroschisis partially covered with collagen scaffold.* ***C.*** *Gastroschisis completely covered with collagen scaffold.*

Neonatal outcome and evaluation

After birth, the regenerated part of the abdominal wall at the site of the incorporated scaffold was macroscopically observed, palpated, measured and photographed. Subsequently the lambs were sacrificed with medetomidine (0.5 mg, intramuscular, Orion pharma, Espoo, Finland) and pentobarbital (60 mg/kg, intracardial).

The abdominal wall of the lambs was opened with broad margins around the site of the former scaffold. The intra- and extra-abdominal parts of the bowel were evaluated for adhesions and fibrous peel formation. The abdominal wall and the bowel, from pylorus to rectum, were taken out.

Histological staining

Tissue samples were taken from the bowel situated outside the abdominal cavity and from bowel inside the abdominal cavity. Additionally, tissue samples were taken from the regenerated tissue at the site of the implanted collagen scaffold and from the bowel lying underneath the site of the implanted collagen scaffold.

The tissue samples were fixed in 10% (v/v) buffered formalin and paraffin-embedded for routine histological processing. Sections (4 μm) were cut and stained with haematoxylin and eosin and Masson's trichrome. The intestinal tissue was examined for changes in the mucosal, submucosal, muscle and serosal layers, and fibrous peel and adhesion formation. Two bowel samples from each fetus were used to assess the thickness of the muscularis propria and the serosa with fibrous peel layer (if present) in three random fields using an ocular micrometer at 100x magnification. The specimens which included the site of the implanted collagen scaffold were examined for evidence of epithelialization, neovascularization, smooth muscle cell ingrowth, signs of inflammation, and degradation of the scaffold.

Data analysis

Data analysis of the bowel measurements was performed with SPSS 16.0 for Windows (SPSS, Chicago, USA), and expressed as mean ± standard deviation. Statistical analysis was performed using the independent samples t-test for equality of means. $P<0.05$ was considered statistically significant.

Results

Animal surgery

Eight of the 12 operated fetuses were born alive (overall survival 67%). Two fetal abortions occurred, one with no distinct reason, one due to a torsion of the eviscerated bowel. Two pregnant ewes were euthanised because of fascial dehiscence 3 weeks after operation. No additional maternal deaths occurred.

Macroscopic results

After birth, 5 lambs presented with a closed abdominal wall and all bowel loops inside the abdominal cavity (lambs 1-5). Two lambs had a partially extra-abdominal bowel which was covered with regenerated skin tissue that replaced the scaffold (lambs 6 and 7). In lamb 8 the scaffold had ruptured and the eviscerated bowel was covered with fibrous peel and very coalescent, like in (lambs with) untreated gastroschisis. An overview of characteristics is given in Table 1.

Table 1. *Characteristics of operated lambs after birth*

Lamb	Location bowel	Complications
1	Intra-abdominal	-
2	Intra-abdominal	-
3	Intra-abdominal	-
4	Intra-abdominal	-
5	Intra-abdominal	Obstructive ileus
6	Largely extra-abdominal	-
7	Small part extra-abdominal	-
8	Extra-abdominal	Gastroschisis

Lambs 1-5 presented with a closed abdominal wall, the bowel was entirely inside the abdominal cavity. From the marking sutures inwards, first normal appearing skin tissue with hair growth was visible. In the centre of the regenerated tissue hyperkeratotic tissue was seen (Figure 2A), and in 2 lambs a small central dry ulcer was found. In 4 of these 5 lambs the bowel showed no abnormalities, and only some minor adhesions to the abdominal wall were seen (Figure 2B). In the other lamb (lamb 5) we saw massive adhesions between bowel loops and with the abdominal wall (Figure 2C). The proximal bowel loops were very dilated. Dissection revealed a small loop with stenosis, which was the cause of the obstructive ileus in this lamb. In all these 7 lambs the skin edges that surrounded the former defect, situated between the area where the scaffold was sutured to the skin and the abdominal wall defect, had formed epidermal inclusion cysts.

Of the 2 lambs in which the bowel was partially outside the abdominal wall, lamb 6 presented with a large extent of extra-abdominal bowel, measuring 7 x 8 x 2 cm (length x width x height) in size. The bowel was covered with skin tissue and appeared to be in a subcutaneous pocket. The skin appeared normal, even with hair growth, with the exception of a small central area (Figure 3A). The bowel was enveloped in a sac of peritoneum which was adhesive to the skin of the abdominal wall (Figure 3B). A 2 cm defect in the abdominal wall was palpable. The extra-abdominal bowel had some minor adhesions, no adhesions were seen inside the abdomen (Figure 3C,D). Lamb 7 presented with less extra-abdominal bowel, measuring 3.5 x 2 x 1 cm. The centre of the regenerated skin, which covered the bowel, was hyperkeratotic, and the surrounding tissue had a ridged configuration (Figure 3E). The extra-abdominal bowel showed moderate adhesions, but the intra-abdominal bowel was without adhesions (Figure 3F). In both lambs no fibrous peel formation was seen on the bowel.

Lamb 8 had a rupture of the scaffold, uncovering the eviscerated bowel. The bowel loops were very coalescent, and showed extensive adhesions and fibrous peel formation (Figure 2D). Inside the abdomen only minor adhesions were observed. Remnants of the scaffold were visible on the abdominal wall.

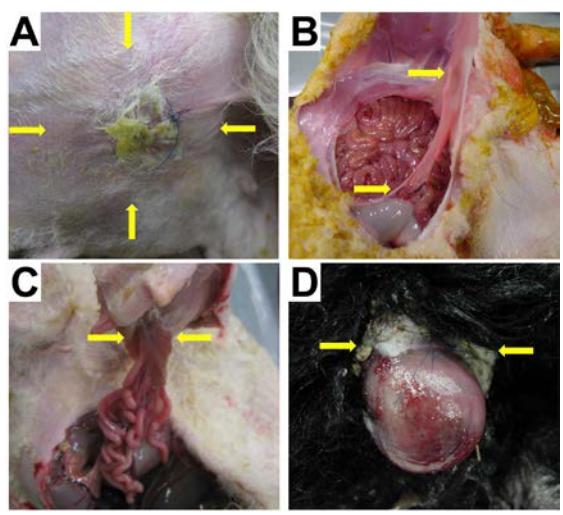

Figure 2. *Macroscopic aspects after birth (lambs 1-5 and 8)* ***A.*** *Showing regenerated skin-like tissue (between arrows) and central hyperkeratinization.* ***B.*** *Minor adhesions after opening the abdominal wall.* ***C.*** *Lamb 5 in which bowel loops were very adhesive, arrows pointing at former defect in abdominal wall.* ***D.*** *Lamb 8 in which an uncovered gastroschisis was found; bowel loops were covered with fibrous peel and adhesive. Arrows pointing at remnants of scaffold.*

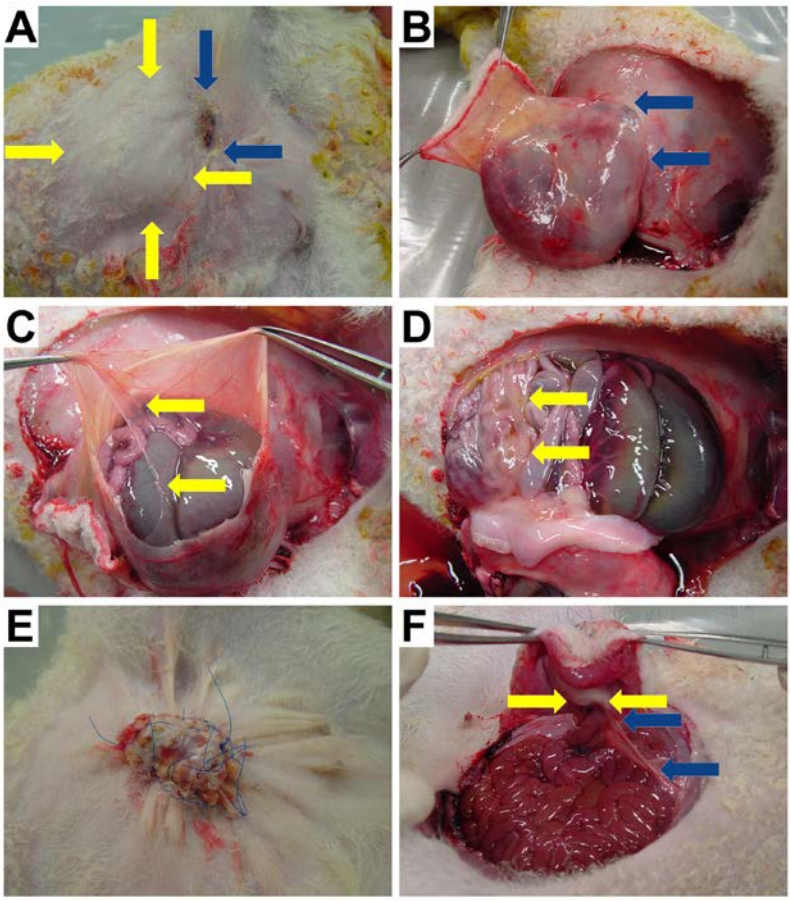

Figure 3. *Macroscopic aspects after birth (lambs 6 and 7).* ***A.*** *Covered gastroschisis in lamb 6. The skin is bulging (yellow arrows) caused by the subcutaneous, partially extra-abdominal, bowel loops. Less mature skin tissue at centre of newly formed tissue (blue arrows).* ***B.*** *After opening the skin tissue, bowel loops enveloped in peritoneum were visible. Defect in the abdominal wall between blue arrows.* ***C.*** *After opening of the peritoneal sac bowel loops were seen, and had only slight adhesions (yellow arrows).* ***D.*** *Intra-abdominally only slight adhesions (yellow arrows) were found as well.* ***E.*** *Lamb 7 presented with only a small part of bowel outside the abdominal wall, hyperkeratotic tissue was covering the bowel.* ***F.*** *After opening the skin, the defect in the abdominal wall was visible (between yellow arrows), and only slight adhesions of the bowel were observed (blue arrows).*

Histological results

Bowel tissue

Normal historical control lambs in our previous study had a serosa of 0.02 ± 0.01 mm and an intestinal muscularis of 0.08 ± 0.03 mm [20].

In lambs 1-6 no histological abnormalities of the bowel tissue were observed. No fibrous peel formation was found, the serosal layer (0.04 ± 0.04 mm) and the intestinal muscularis (0.05 ± 0.02 mm) showed no statistical significant difference in thickness compared to control lambs (p=0.09 and p=0.07 respectively). The submucosa and mucosa appeared normal (Figure 4A). In lamb 7 ischemic necrosis with hemorrhagic infarction was present in the bowel loop with stenosis, and necrosis of the mucosa was observed. In lamb 8 the serosa of the eviscerated bowel was covered with fibrous peel, (0.37 ± 0.05 mm) consisting of deposits of fibrin, degenerated granulocytes and granulation tissue. The fibrous peel was covered with a pseudo-epithelial mesenchymal layer of cells, which seemed to protect the bowel tissue against the amniotic fluid (Figure 4B). Neither prominent inflammation, nor edema, venous dilatation, lymphatic dilatation or signs of ischemia in the bowel tissue were seen. The mucosa appeared normal, with normal slender villi, and the submucosa was normal, without collagen deposits. The intestinal muscularis was not thickened (0.06 ± 0.02 mm), but did show collagen deposition; ganglion cells were normal.

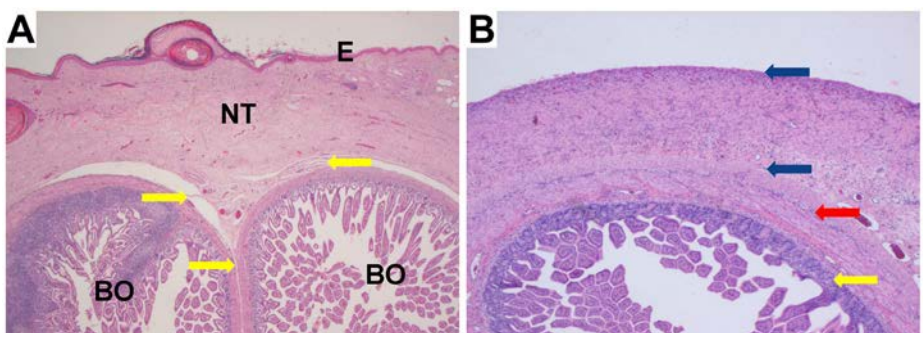

Figure 4. *Histological results of bowel loops.* ***A.*** *Lambs 1-7, showed a normal appearance, with a normal thin serosa (arrows). BO=bowel loop. NT=newly formed tissue. E=newly formed epidermis.* ***B.*** *Lamb 8, showed fibrous peel (between blue arrows), slight thickening of the intestinal muscularis (red arrow) and normal mucosa (yellow arrow). (H&E staining, original magnification x25).*

Abdominal wall tissue

In all lambs, histological examination of the abdominal wall tissue covering the original defect showed that the porous layer of the collagen scaffold was replaced by connective tissue consisting of collagen and fibroblasts. There was a firm connection with the adjacent skin and subcutaneous tissue of the native abdominal wall. In all lambs, the exterior side of the regenerated tissue was covered with skin tissue. The newly formed skin tissue was more mature at the edges, with epithelialization and

appendages including sebaceous glands and hair follicles, compared to the centre (Figure 5A-D). Hyperkeratinization was visible at the centre of this tissue, with a hyperplastic epidermis underneath (Figure 5A,D). Tissue regeneration seemed to occur from the borders of the native tissue to the centre of the newly formed tissue. Neovascularization was seen throughout the entire regenerated tissue. Only a minor chronic inflammatory reaction was observed. The collagen scaffold was largely degraded, except for the less-porous film layer, which was still visible at the inside of the regenerated tissue. In 2 lambs a small ulcer was found, which showed remnants of the scaffold, fibrin deposition, granulocytes and necrosis.

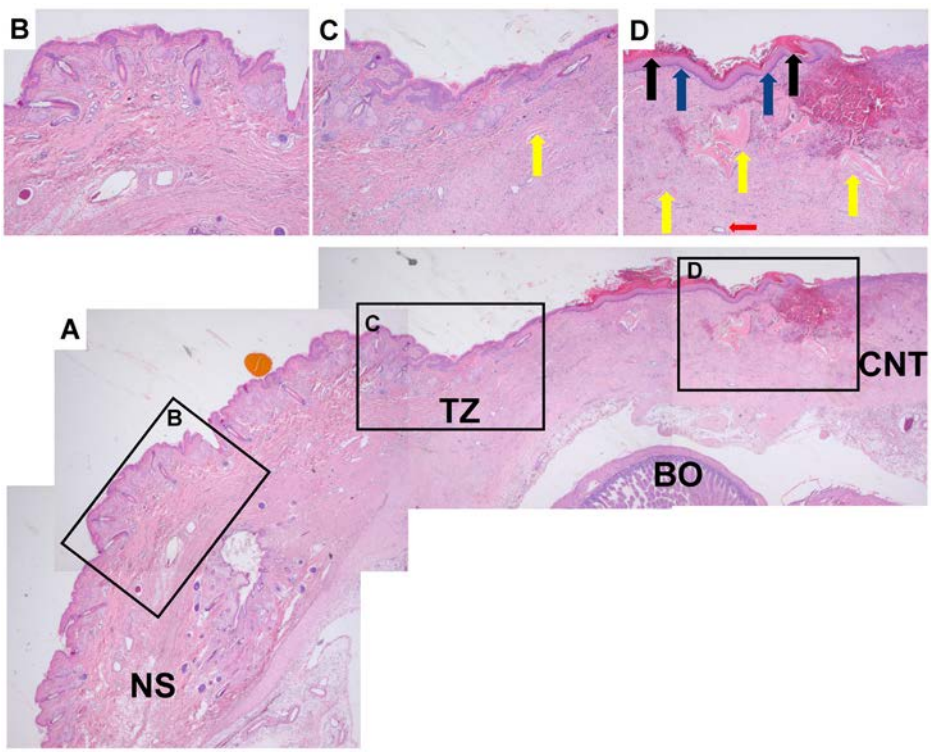

Figure 5. *Histological results of regenerated tissue.* ***A.*** *Overview of regenerated tissue, showing regenerated skin and connective tissue that replaced the scaffold. NS=native skin tissue and subcutaneous tissue. TZ=transition zone between native tissue and newly formed tissue. CNT=central part of newly formed tissue. BO=bowel loop. (H&E, x12.5) B, C and D are insets of A (H&E, x25).* ***B.*** *Native skin tissue with appendages at the surface.* ***C.*** *Transition zone between native tissue on the left side and newly formed tissue on the right side. Remnant of the scaffold (arrow).* ***D.*** *Newly formed skin and subcutaneous tissue at the centre. At the surface hyperkeratinization was visible (black arrows), with hyperplastic epidermis underneath (blue arrows). Remnants of the scaffold were observed in the centre of the tissue (yellow arrows). Capillary (red arrow).*

Discussion

The mortality rate in gastroschisis patients is reported to be 4%-12.5%, but postnatal complication rates up to 79% have been reported [1-4]. Damage to the bowel is proposed to occur during late pregnancy, due to constriction of the bowel at the site of the abdominal wall defect and exposure to the amniotic fluid [5]. Gastroschisis can be detected in early pregnancy [15], which offers the potential option to salvage the bowel tissue with fetal therapy.

Stephenson *et al.* repaired an experimental gastroschisis in sheep 25 days after creation, by replacing the bowel into the abdomen and subsequent surgical closure of the abdominal wall. They reported reversal of damage to the bowel visible at 100 days of gestation, with a normal appearance of the bowel at term (135 days) [22]. Langer *et al.* surgically created a gastroschisis in fetal lambs at 80 days of gestation, and performed repair at 120 days of gestation, involving relief of bowel constriction and coverage of the eviscerated bowel with a silastic sheet. In the repair group a partial reversal of damage to the bowel was observed at histological evaluation and in bowel contractility studies after birth [6].

In previous work, we used this fetal lamb model for gastroschisis, and performed an immediate repair by suturing the same collagen scaffold as was used in the present study into the abdominal wall defect. Instead of leaving the eviscerated bowel out of the abdominal cavity, as in the present study, in this previous study the eviscerated bowel was replaced into the abdominal cavity before closure of the abdominal wall. The scaffold was replaced by skin tissue and connective tissue, with good neovascularization and ingrowth of smooth muscle cells. No abnormalities of the underlying bowel were seen [20]. In other lambs the bowel had been left eviscerated. In the latter group the bowel resembled human gastroschisis on macroscopic evaluation, i.e. extensive adhesions and fibrous peel formation, and histological evaluation, i.e. a normal mucosa and submucosa, slight thickening of the intestinal muscularis with collagen deposition and fibrous peel formation. The results in this group were comparable to the lamb with the ruptured scaffold in the present study.

In the current study we covered the eviscerated bowel loops with the previously used molecularly defined, biocompatible and biodegradable dual-layer scaffold of highly purified bovine type I collagen. By covering the eviscerated bowel we aimed to prevent fibrous peel and adhesion formation by protecting the bowel against toxic products in the amniotic fluid. Covering the bowel loops instead of closure of the abdominal wall after replacing the bowel loops into the abdominal cavity, as in the previous study [20], will simplify the prenatal operation. For prenatal replacement of the bowel loops into the abdominal cavity the narrow abdominal wall defect needs to be enlarged by a full-thickness incision, and the vulnerable bowel loops, which can already be edematous, thickened and matted, have to be massaged back into the probably already hypoplastic abdominal cavity [22]. These

potentially harmful actions will not be necessary when only covering the eviscerated bowel loops, although an incision in the abdominal wall may still be needed to relieve constriction at the abdominal wall defect. In addition, by using the dual-layer collagen scaffold we aimed to induce cell growth into the scaffold, which ultimately could lead to skin or abdominal wall formation replacing the scaffold, and coverage of the vulnerable eviscerated bowel loops. This would improve postnatal surgical repair when still needed.

The collagen scaffold was sutured into a surgically created skin wound 3 mm from the edges of the abdominal wall defect. This strategy was determined by two considerations. First, in this way the scaffold was not tightly placed around the base of the eviscerated bowel, which is far smaller than the widest diameter of the total package of bowel loops due to the small defect in the abdominal wall. Second, a fresh wound is necessary to induce the repair mechanisms and cellular ingrowth into the scaffold.

In most of the lambs the bowel had returned inside the abdominal cavity at birth. The likely explanation is that slight shrinkage of the scaffold and the growth of the bowel loops has increased pressure in the pocket, allowing the bowel loops to be gradually replaced into the abdominal cavity, mimicking postnatal silo closure in gastroschisis. Skin tissue had formed, and closed the abdominal wall defect. In 2 lambs the bowel loops were still partially outside the abdominal wall, however covered by regenerated skin tissue. In these 2 lambs skin tissue had replaced the collagen scaffold, the extra-abdominal part of the bowel was lying underneath in a subcutaneous pocket. In all but one lamb the bowel appeared normal, without peel formation and with only minor adhesions between bowel loops or with the abdominal wall. The exception was a lamb in which the entanglement of the bowel loops due to adhesions had led to ischemic necrosis and an obstructive ileus. Histology confirmed the normal appearance of the covered bowel loops after birth, without fibrous peel formation or intestinal muscularis thickening. The porous part of the scaffold had been replaced by skin-like tissue and connective tissue.

The film layer was not degraded at birth, and was still visible at the inside of the regenerated tissue. Chemical crosslinking of the collagen and the addition of the film layer, to create a dual-layer scaffold, gave the porous scaffold extra tensile strength [20]. The film layer has less porosity, so cells will not grow into this part of the scaffold. However, due to the low porosity it is presumed to be less or impermeable for toxic products in the amniotic fluid. Additionally, it has higher strength capabilities, and, due to the lower degradability of this layer, the strength of the dual-layer scaffold will be maintained for a longer period. Although the tensile strength of the scaffold was improved, the scaffold had ruptured in one lamb, in which the bowel loops were left uncovered and fibrous peel formation and adhesions were observed. In future research we will combine the collagen scaffold with a degradable polymer for further improvement of the tensile strength. In addition, improved skin regeneration might be obtained with the application of growth factors to the collagen scaffold, as

we demonstrated in a previous study [19].
Prenatal coverage of bowel in gastroschisis with a collagen scaffold is possible in mid-gestation fetal lambs, protecting the bowel loops against toxic waste products in the amniotic fluid. The collagen scaffold is degraded and replaced by skin and connective tissue at birth. In most of the lambs the bowel was repositioned into the abdominal cavity, which may make an extra operation after birth unnecessary. When the bowel still is partially outside the abdominal cavity, like in 2 lambs in the present study, a closure operation will still be needed. However, primary closure may be more straightforward because the bowel will not be thickened or dilated. In addition, this technique provided newly formed skin tissue that can be used during the repair procedure. An obstructive ileus due to adhesions was the only major complication we observed in one lamb, emphasizing the need for careful follow-up when using this technique.
Currently, the major disadvantage of fetal surgery is the risk of complications leading to premature delivery [23,24]. This risk may currently not outweigh the possible benefit for the child born with gastroschisis. However, if intrauterine coverage of the bowel in gastroschisis is possible with an endoscopic approach, it might become a minimal invasive treatment option for patients with this severe congenital anomaly.

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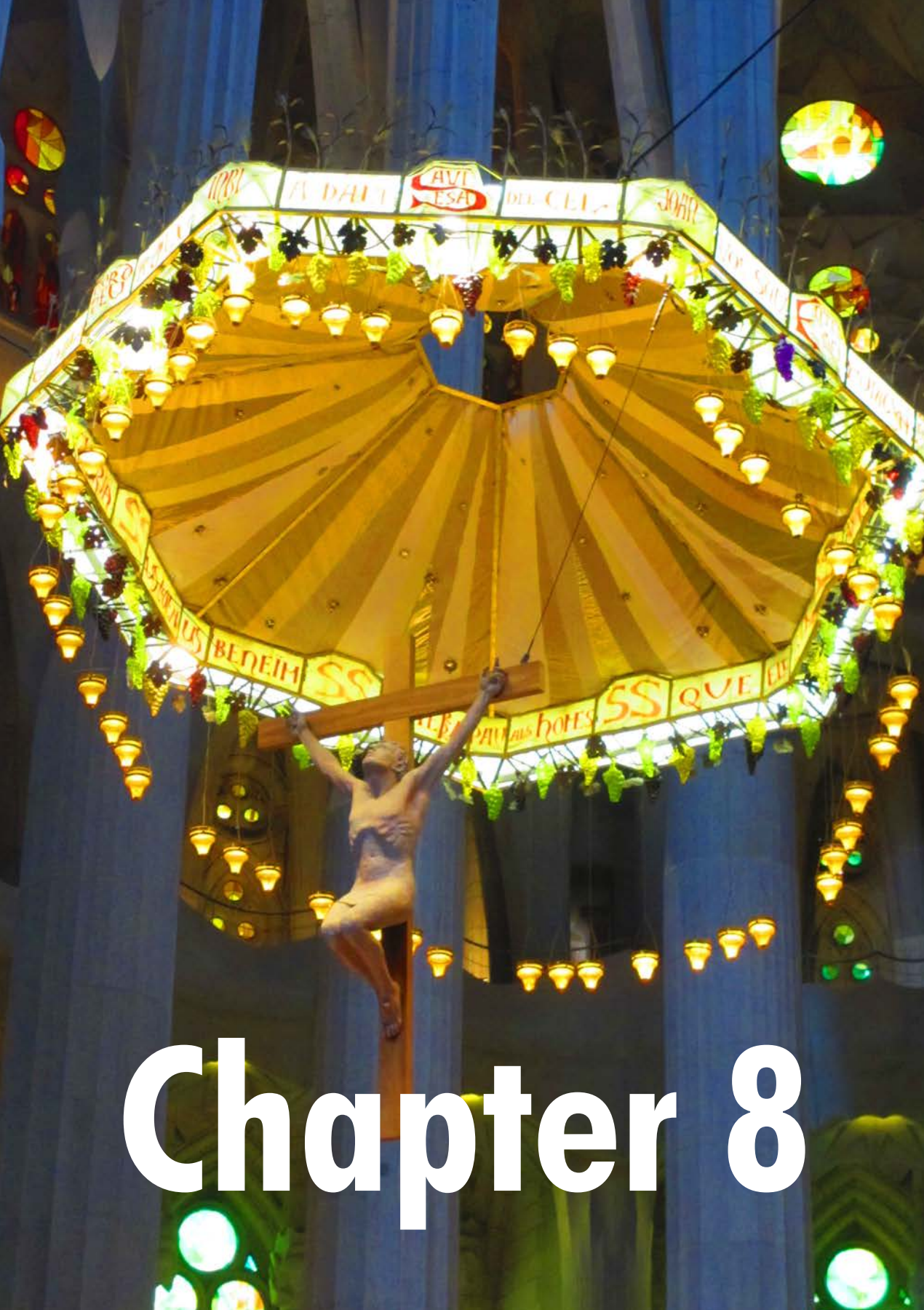

Chapter 8

Summary and Future Perspectives

Samenvatting en Toekomstvisie

Summary

Introduction
This thesis deals with personal health care opportunities for children with a congenital anomaly using 'Regenerative Medicine'. Treatment possibilities for children with a dysfunctioning bladder are discussed in chapters 2 to 5. Tissue engineering for children with abdominal wall defects such as gastroschisis is discussed in chapters 6 and 7.
Children with congenital anomalies such as bladder exstrophy, myelomeningocele or posterior urethral valves may develop small-capacity bladders with low compliance and high intravesical pressures. A bladder augmentation may be needed to create adequate reservoir function and low intravesical pressure to preserve upper urinary tract function. Bladder augmentation is usually performed with gastrointestinal tissue. However, this is not always sufficiently available and can lead to metabolic disturbances, infections, excessive mucus production, stone formation, perforation and even malignancies. In our centre complications were seen in 49% of operated children. Alternatives for the use of gastrointestinal tissue would therefore be desirable.
Gastroschisis is an abdominal wall defect, in which a large part of the bowel is herniated outside the abdominal cavity. Damage to the bowel is caused by constriction at the site of the abdominal wall defect and/or the toxic effect of the amniotic fluid. Mortality is 4-12.5%, and morbidity can be severe. Primary closure of the abdominal wall can be problematic because the abdominal cavity is relatively hypoplastic and the bowel volume is enlarged. Gastroschisis can be detected in early pregnancy, offering the opportunity to salvage the bowel tissue using fetal therapy. Tissue-engineered constructs could be a solution for the operative closure of these defects.
Tissue engineering is part of the rapidly growing field of 'Regenerative Medicine' and aims at repair or replacement of damaged or removed organs by inducing regenerative processes. A construct or scaffold is used consisting of a natural material (predominantly collagen) or synthetic polymers, or a combination of these, forming an artificial extracellular matrix (ECM) which serves as a 'skeleton' to support cell growth. After implantation these constructs will be replaced by autologous tissue. Structural modifications or the addition of autologous cells could improve the quality of regenerated tissue. Furthermore, the addition of growth factors involved in proliferation, migration and differentiation of several cell types may help to modulate the regenerative process.

Chapter 2

In this chapter we describe the creation of a fetal sheep model to mimic bladder changes in patients with bladder exstrophy, and evaluated histological alterations in the different layers of the bladder. Additionally, we studied fetal bladder tissue regeneration by the immediate reconstruction of the bladder defect using a collagen scaffold.

At 79 days' gestation, a hysterotomy on the ewe and a laparotomy on the fetus were performed. In group 1 the fetal bladder was opened and sutured to the abdominal wall. In group 2 a dual-layer collagen scaffold was sutured to the opened bladder and the abdomen was closed. Lambs were born at term (140-147 days), sacrificed, and a cystogram and histological examination were performed.

The survival rate of 92% was high and the model was highly reproducible. The histological results of the bladders in group 1 showed remarkable resemblance with the changes in bladder tissue of human congenital bladder exstrophy. We found distinct changes of the mucosa in 4 out of 5 lambs. Ulceration of the urothelial layer was seen in 3 lambs, with granulation tissue and chronic reactive inflammation; squamous metaplasia was present in 1 lamb. Submucosal fibrosis was apparent and the number of capillaries was increased in all lambs. The detrusor muscle showed atrophy and fibrosis in the inner layer in 4 lambs. In group 2, histological examination revealed regeneration of urothelium, angiogenesis, and the ingrowth of smooth muscle cells and nerve fibres. The porous layer of the collagen scaffold was largely degraded.

This animal model can be useful for further studies on fetal bladder development in bladder exstrophy, and to evaluate new approaches to salvage or regenerate bladder tissue in this anomaly. This study showed that fetal tissue engineering of the bladder was feasible in an animal model. The major drawback of fetal surgery at present is the risk of complications leading to premature delivery. Improvements are needed, perhaps by using fetoscopy, for the benefits to outweigh the risks of fetal therapy, particularly for non-lethal anomalies. Furthermore, fetal repair of a bladder exstrophy would still be highly challenging.

Chapter 3

Preclinical animal studies have generally been performed in healthy bladders. However, translation to patients is hampered by observations that cultured urothelial and smooth muscle cells from patients with a neuropathic bladder or bladder exstrophy behave dissimilar from normal cells, which may have implications for tissue engineering. Indeed, two animal studies demonstrated that tissue engineering of diseased bladder led to much more fibrosis, less smooth muscle cells and poor functionality. The animal model presented in chapter 2 was used to represent a diseased bladder because it closely mimics the naturally occurring bladder exstrophy.

In chapter 3 we investigated whether the capability of diseased bladder to regenerate bladder tissue was comparable to bladder tissue regenerated in healthy bladder.
For this purpose we reconstructed the bladder of lambs in which a bladder exstrophy-like lesion was prenatally created using a collagen scaffold, one week after birth. This method was compared to suturing a scaffold in the bladder of healthy lambs one week after birth. Functional and histological evaluation was performed after 1 and 6 months.
Video urodynamics showed no difference in capacity or compliance of the bladders between both methods at 1 and 6 months, and histological evaluation revealed no major differences. Squamous differentiation of the urothelium was present at the borders between native and regenerated tissue in the diseased bladders 1 month after reconstruction. This might suggest the presence of urothelial changes in regenerated tissue of diseased bladders at early time points. However, at 6 months the urothelium of the regenerated tissue appeared normal, implicating a reversible effect.
We showed that bladder tissue engineering with a highly porous collagen scaffold is possible in a diseased animal model. In this animal model for bladder exstrophy we found comparable bladder regeneration to healthy bladder, resulting in tissue of good quality.

Chapter 4
Growth factors are involved in proliferation, migration and differentiation of several cell types. Heparin can be bound to collagen scaffolds, which improves growth factor binding to the scaffold and decreases proteolytic degradation of growth factors resulting in a sustained release system. Vascular endothelial growth factor 165 (VEGF165) is an important factor in angiogenesis. The combination with fibroblast growth factor 2 (FGF2) enhances blood vessel formation and maturation. These growth factors were coupled to collagen scaffolds loaded with heparin, in combination with heparin-binding epidermal growth factor (HB-EGF) which is involved in urothelial regeneration.
In this study we evaluated the effect of incorporation of growth factors VEGF165, FGF2 and HB-EGF in a collagen-heparin scaffold on bladder tissue regeneration and functionality. This scaffold was applied in the bladder exstrophy model (COLGF-group), and results were compared with those of a scaffold without growth factors in the same model (COL-group), and with reconstruction without the use of a scaffold (primary closure) (PC-group). Functional and histological evaluation was performed after 1 and 6 months.
Histology showed improved ingrowth of urothelial cells in lambs in the COLGF-group, with confluent multi-layered and well-differentiated urothelium after 1 month, compared to an interrupted layer of urothelium in lambs in the COL-group. Angiogenesis was more profound in the COLGF-group compared to the COL-group

at 1 month. Smooth muscle cell ingrowth was improved in the COLGF-group at both time points. The improved regeneration of bladder tissue did not lead to statistically significant increment of urodynamic results.
A collagen scaffold incorporated with growth factors VEGF165, FGF2 and HB-EGF enhanced tissue regeneration in a diseased large animal model. This resulted in tissue of good quality involving all layers of the bladder.

Chapter 5
The ileal conduit is considered the current standard urinary diversion for patients with bladder cancer and some pediatric patients with severe congenital anomalies. Complications are mainly related to the use of gastrointestinal tissue. Tissue engineering may be the technical platform to develop alternatives to gastrointestinal tissues. We developed a collagen-polymer conduit and evaluated its applicability for urinary diversion in a porcine model.
Tubular constructs of 12 cm in length and 15 mm in diameter were prepared from bovine type I collagen and Vypro® II synthetic polymer mesh. Characterized tubes were sterilized, seeded with and without primary porcine bladder urothelial cells, and implanted as an incontinent urostomy using the right ureter in 10 female Landrace pigs. At 1 month, the newly formed tissue structure was functionally and histologically evaluated.
The survival rate was 80% with 1 related (complete stenosis of stoma-side) and 1 unrelated death. Stoma-side stenosis was present in all animals. At 1 month the collagen was resorbed and a retroperitoneal tube was formed that withstood 40 cm H_2O pressure. In 5 cases the tube functioned as a urostomy; loopograms revealed stenosis at the ureteral anastomosis in 3 pigs, of which 2 had leakage. In 4 cases polymer mesh was found in the lumen of the conduits that was neither degraded nor incorporated in surrounding tissue. In all pigs the right upper urinary system showed hydroureteronephrosis. Histological analysis revealed a moderate immune response and angiogenesis. Urothelial cells were scarce in the construct lumen, but a continuous urothelial lining was seen at the area of the ureteral anastomosis. The polymer mesh provoked fibroblast deposition and tissue contraction. No major differences were observed between cellular and acellular constructs.
The tubular construct formed a retroperitoneal tube that functioned as a urinary conduit in most cases. Nevertheless, improvements of this technique need to be achieved before clinical application is possible, perhaps by incorporating another degradable polymer with improved biocompatibility and by everting the stoma to prevent stoma-side stenosis. Improved large tubular scaffolds may generate alternatives to gastrointestinal tissue for urinary diversion.

Chapter 6

Primary closure of severe congenital abdominal wall defects can be complicated by hypoplasticity of the abdominal cavity and the enlarged volume of the bowel caused by edema and peel formation. In a fetal sheep model for gastroschisis we therefore evaluated regeneration of the abdominal wall using a dual-layer collagen scaffold, and its protective effect on the bowel.

In 14 fetal lambs, a gastroschisis was created by opening the abdominal wall and eviscerating the bowel at 79 days' gestation. In one group the eviscerated bowel was left uncovered; in a second group the bowel was repositioned and the defect was closed by suturing a collagen scaffold into the abdominal wall. A cesarean section was performed at 140 days' gestation, and macroscopic and histological evaluation was performed.

Eleven lambs (79%) were born alive. In the 5 lambs with a gastroschisis, the eviscerated part of the bowel was coalescent, showed extensive adhesions, and was covered by fibrous peel. In the other lambs the abdominal wall was closed, with a firm connection to the native abdominal wall. In 1 lamb a small hernia was palpable. Minor or no adhesions of the bowel, and no peel formation were observed. The scaffold was largely degraded and replaced by connective tissue with collagen and fibroblasts, angiogenesis, and scattered muscle cells. Skin tissue was more mature at the edges, with epithelialization and adnexal differentiation, compared to the centre.

Abdominal wall tissue regeneration using a collagen scaffold was feasible in fetal lambs, resulting in a closed abdominal wall at birth and regenerated skin and connective tissue with angiogenesis and muscle cells. Immediate closure of the gastroschisis strongly diminished or prevented bowel adhesions and peel formation.

Chapter 7

In fetuses with gastroschisis the eviscerated bowel is affected by toxic products in the amniotic fluid and constriction at the site of the abdominal wall defect. Fetal coverage of the eviscerated bowel may prevent further secondary damage to the bowel. The aim of this study was to cover the eviscerated bowel in gastroschisis with a collagen scaffold to protect the bowel, and to induce cell growth into the scaffold, which might lead to skin or abdominal wall formation.

In 12 fetal lambs a gastroschisis was surgically created at 79 days' gestation. A dual-layer type I collagen scaffold was sutured into the skin of the abdominal wall around the defect, covering the eviscerated bowel. Macroscopic and histological evaluation was performed after caesarean section at 140 days' gestation.

Survival was 67%. In 7 of 8 surviving lambs the bowel was covered after birth, in one case the scaffold had ruptured. In 5 lambs the bowel was found to be repositioned into the abdominal cavity, in 2 lambs it was still partially outside. In 6 lambs only minor adhesions of bowel loops were observed, one lamb had extensive

adhesions resulting in an obstructive ileus. No fibrous peel formation was seen. Connective tissue with collagen, fibroblasts and angiogenesis replaced the scaffold, and was covered by skin tissue. Skin tissue was more mature at the edges, with epithelialization and adnexal differentiation, compared to the centre of regenerated tissue.
Prenatal coverage of the bowel in gastroschisis with a collagen scaffold is feasible in fetal lambs, and strongly diminished damage to the bowel. Skin and connective tissue replaced the scaffold. Covering the bowel loops instead of closure of the abdominal wall after replacing the bowel loops into the abdominal cavity, as in chapter 6, simplifies the prenatal operation. Postnatal reconstruction will be easier as well, or may even be unnecessary.

Future Perspectives

For a significant impact on personalized treatment options in the field of 'Regenerative Medicine', future studies should focus on the use of larger scaffolds to obtain clinically significant augmentation of the bladder. However, tissue regeneration in the center of such large scaffolds can be hampered by late onset of angiogenesis in this area. Due to the lack of oxygen and nutrition delivery to the cells and inadequate removal of waste products, tissue regeneration will be impaired, resulting in fibrotic scar tissue or even necrosis. Since the amount of oxygen, required for cell survival, is limited to a diffusion distance of approximately 150-200 µm from the supplying blood vessel, angiogenesis needs to be improved into these large scaffolds.
Omentum is highly vascularized, and can be wrapped around or over a tissue-engineered construct to enhance ingrowth of blood vessels into the construct originating from the omentum. We used this technique in chapters 3 and 4. A phased reconstruction may improve tissue regeneration when using a construct seeded with autologous cells. First, the construct is wrapped in omentum [1], and during a second operation sutured to the bladder. Between these operations, angiogenesis into the construct can occur, hereby creating an improved microenvironment for the seeded cells, before cells are exposed to urine, which may have a deleterious effect on these cells.
Another approach to improve angiogenesis in scaffolds is the use of growth factors, as confirmed in chapter 4. Larger scaffolds loaded with growth factors need to be tested for their capacity to regenerate bladder tissue of good quality throughout the whole implanted construct. More information is needed about the best combinations and concentrations of growth factors for each specific tissue. Growth factors such as platelet-derived growth factor-BB (PDGF-BB), hepatocyte growth factor (HGF) or insulin-like growth factor 1 (IGF-1) are known to induce smooth muscle proliferation, and should be investigated, probably in combination with the growth factors used

in chapter 4. On the basis of the results, a scaffold with a specific 'cocktail' of growth factors can be produced, tailored for the tissue to be regenerated.
Perhaps the combination of growth factors together with autologous cells on a scaffold could enhance tissue regeneration even more. Autologous cells should be seeded on scaffolds incorporated with growth factors, may be cultured for several days on the scaffold, after which the construct can be implanted. This theory should first be investigated *in vitro*, to evaluate the influence of culturing cells on this scaffold on the amount and activity of the incorporated growth factors, before *in vivo* studies can be performed.
Another approach to overcome the problem of angiogenesis in large scaffolds is currently investigated by our group. Instead of one large scaffold, we use several smaller scaffolds to reach the same surface area of regenerated tissue. The vascularized border of the native tissue will be closer to the centre of the scaffold, hereby shortening the time until this area will be vascularized.
Schultheiss *et al.* presented an interesting approach to increase vascularization, which may be applicable for bladder tissue engineering [2]. A porcine small bowel segment was isolated and decellularized. The matrix was seeded with autologous urothelial and smooth muscle cells. Vessels were resurfaced with endothelial progenitor cells by perfusing these cultured cells. The duration of circuit patency was determined by continuous perfusion with a peristaltic perfusion pump in later studies of this research group [3]. Hereby, a construct was created with an intact circuit of vessels, which were anastomosed during implantation in a porcine model. The construct was viable and without thrombus formation when evaluated after 3 hours [2]. An experiment in 1 human patient showed the viability of this construct for 1 week, however only endothelial cells were seeded in this construct [3].
Turner *et al.* used another tissue engineering technique to perform a cystoplasty [4]. They cultured sheets of autologous urothelium harvested with a biopsy. During the operation a segment of sigmoid was isolated and surgically de-epithelialised. This segment was covered with the urothelial sheet and subsequently implanted in the bladder. The sheet was secured to the bowel segment with a vicryl mesh and a silicone vesical balloon. This resulted in a construct lined with confluent urothelium, without fibrosis, mucus, calculi or colonic regrowth after 3 months.
The tissue engineered tubular construct presented in chapter 5 should be improved before it can be used as an incontinent conduit. Vascularization may be improved by wrapping the construct in omentum or peritoneum and needs to be tested. Furthermore, other degradable polymers with improved biocompatibility should be evaluated. To overcome the stoma-side stenosis everting the tube at the skin side may be a solution.

The 'Achilles heel' of fetal surgery at present is the risk of complications leading to premature delivery. Improvements of the technique, perhaps by using minimal invasive treatment by laparoscopic intervention on a fetus (fetoscopy), are necessary before the benefits of fetal therapy outweigh the risks. This particularly accounts for anomalies like gastroschisis which are generally non-lethal.
First attempts to repair a surgically created gastroschisis in a fetal lamb using fetoscopy failed. The bowel volume was enlarged by the inflammatory fibrotic peel and distended bowel loops, resulting in severe hemodynamic compromise of the fetus when the bowel was returned into the relatively hypoplastic abdominal cavity. This may be overcome by using a scaffold for fetoscopic closure, as described in chapter 6, with which the abdominal wall is augmented, and intra-abdominal pressure will rise less than by primary closure. As described in chapter 7, coverage of the eviscerated bowel is an option in difficult cases when repositioning the bowel into the abdominal cavity is not possible. These techniques need to be tested using fetoscopy.
Improvement of the material may be desired for fetal abdominal wall defect repair. In chapter 6 and 7 we found 1 lamb with a ruptured scaffold and 1 lamb with a small hernia, probably caused by insufficient tensile strength of the dual-layer scaffold. Combining the collagen scaffold with a degradable polymer could be a solution.
Incorporating growth factors in the scaffold may also lead to further improved abdominal wall regeneration. In previous research we found that the addition of VEGF and FGF2 to a collagen scaffold had advantageous effects on fetal skin regeneration [5]. Wound contraction decreased, angiogenesis improved, and fewer myofibroblasts were observed. The addition of growth factors to a scaffold may result in faster closure of the wound and perhaps improves ingrowth of skeletal muscle cells.

These refinements should ultimately result in products which can be tested in clinical trials. However, many considerations need to be taken into account before tissue-engineered products can be used in clinical trials. Important issues were recently described by Oerlemans *et al.* [6]. 1) The tissue-engineered product has a multifaceted complexity since it has a certain amount of variability, a dynamic interaction with the body, and due to the influence on surrounding tissue the process cannot be undone. 2) Testing the product is difficult due to the lack of a gold standard established in randomized clinical trials. 3) The patient is a child and has a large life span, exposing the patient to possible long-term complications, e.g. malignant degeneration. Furthermore, the informed consent needs to be obtained by the parents, and the decision may have consequences for the far future. The treatment is highly complex and has different goals and risks than conventional surgical procedures, resulting in difficult decision making for lay people.

The authors suggest an adjusted plan for testing tissue-engineered products for children. Animal models used need to be optimal representations of the situation in humans. Therefore, animal models which closely mimic the 'diseased' situation in humans need to be used if available or developed. Performing a systematic review is an 'evidence-based' tool to identify the best suitable animal model [7,8].
The first step after animal studies should be small-scale expert case series with actual patients. Potential risks need to be minimized and benefits need to be maximized before this step. Products need to be produced under Good Manufacturing Practice (GMP) guidelines, in GMP authorized facilities (e.g. clean rooms) under standardized conditions and with controlled sterilization methods. Next, trials should be conducted according to Good Clinical Practice guidelines for clinical trials. Both the production and testing of the products have to be performed according to the European Medicines Agency (EMA) regulations of the European Union on 'Advanced therapies': 'tissue-engineered products' or 'combined advanced-therapy medicines' [9].
Informed consent should consist of a comprehensible explanation with possible benefits and risks on the short- and long-term. The next step should be the evaluation of the product on larger scale, in different teams of several expert centers based on a uniform protocol. Subsequently, large clinical trials should prove superiority of the product over conventional treatment.

Translational medicine tries to make the translation from 'bench to bedside', by implementing products developed in laboratories into clinical practice. In the EuroSTEC project large efforts have been made in this field for soft tissue engineering by support of the European Union [10]. Hopefully the treatment techniques described in this thesis can result in improved treatment modalities for children with severe congenital anomalies within several years.

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Samenvatting

Introductie

Dit proefschrift behandelt mogelijkheden voor gepersonaliseerde gezondheidszorg voor kinderen met een aangeboren afwijking, met behulp van 'Regeneratieve geneeskunde'. Behandelingsmogelijkheden voor kinderen met een disfunctionerende blaas worden besproken in de hoofdstukken 2 tot en met 5. Tissue engineering van de buikwand voor kinderen met buikwandafwijkingen zoals gastroschizis, wordt besproken in hoofdstuk 6 en 7.

Kinderen met aangeboren afwijkingen, zoals blaasextrofie, myelomeningocele of posterieure urethrakleppen, kunnen een blaas met lage capaciteit en compliantie en hoge intravesicale drukken ontwikkelen. Een blaasaugmentatie kan nodig zijn om een adequate reservoirfunctie en lage intravesicale druk te verkrijgen, om hiermee de functie van de hogere urinewegen te behouden. Een blaasaugmentatie wordt meestal uitgevoerd met gastro-intestinaal weefsel. Het gebruik van gastro-intestinaal weefsel kan echter leiden tot metabole stoornissen, infecties, overmatige slijmproductie, steenvorming, perforatie en zelfs maligniteiten. Daarnaast is niet altijd voldoende darmweefsel beschikbaar. In ons centrum werden bij 49% van de kinderen met een blaasaugmentatie middels gastro-intestinaal weefsel, complicaties gezien. Alternatieven voor het gebruik van gastro-intestinaal weefsel zijn dus zeer wenselijk.

Gastroschizis is een buikwanddefect waarbij een herniatie van een groot deel van de darm buiten de buikholte aanwezig is. Schade aan de darm wordt veroorzaakt door vernauwing op de plaats van het buikwanddefect en/of het toxische effect van het vruchtwater. De mortaliteit is 4-12,5%, en de morbiditeit kan ernstig zijn. Primaire sluiting van de buikwand kan problematisch zijn doordat de buikholte relatief hypoplastisch is en het darmvolume is vergroot. Gastroschizis kan vroeg in de zwangerschap worden gedetecteerd. Dit biedt de mogelijkheid om het darmweefsel met foetale therapie te behouden. 'Tissue-engineered' producten zouden een oplossing kunnen zijn voor de operatieve sluiting van deze defecten.

'Tissue engineering' maakt deel uit van het snel groeiende specialisme 'Regeneratieve geneeskunde' en is gericht op herstel of vervanging van beschadigde of verwijderde organen door het induceren van regeneratieve processen. Een construct of scaffold, bestaand uit natuurlijk materiaal (overwegend collageen) of synthetisch polymeer of een combinatie daarvan, wordt toegepast als kunstmatige extracellulaire matrix (ECM), dat als een 'skelet' voor celgroei fungeert. Na implantatie zullen deze constructen worden vervangen door autoloog weefsel. Structurele aanpassingen aan de scaffold, of de toevoeging van autologe cellen hieraan, zouden de kwaliteit van het geregenereerde weefsel kunnen verbeteren. Bovendien kunnen groeifactoren, betrokken bij proliferatie, migratie en differentiatie van diverse celtypen, worden toegevoegd om het regeneratieve proces te moduleren.

Hoofdstuk 2
In dit hoofdstuk beschrijven we de ontwikkeling van een foetaal schapenmodel dat de veranderingen van de blaas bij patiënten met blaasextrofie nabootst, alsmede de evaluatie van de histologische veranderingen in de verschillende lagen van de blaas in dit model. Daarnaast werd regeneratie van foetaal blaasweefsel onderzocht, waarbij een blaasdefect direct na het aanbrengen hersteld werd door inhechting van een dubbellaags collageen scaffold.
Bij 79 dagen dracht werd een hysterotomie bij de ooi en een laparotomie bij de foetus verricht. In groep 1 werd de foetale blaas geopend en aan de buikwand gehecht. In groep 2 werd een collageen scaffold in de geopende blaas gehecht en werd de buik gesloten. Lammeren werden à terme geboren (140-147 dagen) en geofferd, waarna een cystogram en histologisch onderzoek werden uitgevoerd.
Het overlevingspercentage van 92% was hoog en het model was goed reproduceerbaar. Histologisch onderzoek van het blaasweefsel in groep 1 toonde een opmerkelijke gelijkenis met de veranderingen in blaasweefsel bij blaasextrofie in mensen. Bij 4 van de 5 lammeren vonden we veranderingen van het slijmvlies. In 3 lammeren werd ulceratie van de urotheliale laag in combinatie met granulatieweefsel en chronische reactieve ontsteking gezien; plaveiselcelmetaplasie was bij 1 lam aanwezig. In alle lammeren waren submucosale fibrose en een verhoogd aantal capillairen zichtbaar. Bij 4 lammeren waren atrofie en fibrose in de binnenste laag van de musculus detrusor zichtbaar. Histologisch onderzoek in groep 2 liet regeneratie van urotheel, angiogenese en ingroei van gladde spiercellen en zenuwvezels zien. De poreuze laag van de collageen scaffold was grotendeels afgebroken.
Dit diermodel biedt de mogelijkheid aanvullend onderzoek naar foetale blaasontwikkeling bij blaasextrofie te doen en nieuwe behandelingsmogelijkheden voor regeneratie of het behouden van blaasweefsel bij deze afwijking te evalueren. Deze studie toonde in een diermodel aan dat foetale regeneratie van blaasweefsel mogelijk is. Het grote nadeel van foetale chirurgie is het risico op complicaties die leiden tot vroeggeboorte. Verbeteringen zijn nodig, bijvoorbeeld door gebruik van 'fetoscopy', voordat de voordelen van foetale therapie opwegen tegen de nadelen, met name voor niet-lethale anomalieën. Bovendien blijft foetaal herstel van een blaasextrofie daarbij nog steeds zeer uitdagend.

Hoofdstuk 3
Preklinische dierstudies naar blaasregeneratie zijn over het algemeen uitgevoerd bij gezond blaasweefsel. Vertaling naar patiënten wordt echter belemmerd doordat gekweekte urotheelcellen en gladde spiercellen van patiënten met een neuropathische blaas of blaasextrofie zich afwijkend gedragen ten opzichte van normale cellen, wat gevolgen kan hebben voor weefselregeneratie. Bij twee dierstudies werd inderdaad aangetoond dat 'tissue engineering' van ziek blaasweefsel tot veel fibrose, minder gladde spiercellen en slechte functionaliteit

leidt. Het in hoofdstuk 2 beschreven diermodel werd gebruikt als model voor ziek blaasweefsel, aangezien het een blaasextrofie bij de mens nauwkeurig nabootst. In hoofdstuk 3 onderzochten we of ziek blaasweefsel leidt tot vergelijkbare blaasweefsel regeneratie als gezond blaasweefsel, na sluiting met een collageen scaffold.
Hiervoor werd de blaas van lammeren, waarbij prenataal een blaasextrofie-achtige laesie prenataal werd gemaakt, een week na geboorte gereconstrueerd met behulp van een collageen scaffold. De functionele en histologische resultaten na 1 en 6 maanden werden vergeleken met die waarbij een week na geboorte een scaffold in de blaas van gezonde lammeren werd gehecht.
Video urodynamica toonde geen verschil in blaascapaciteit of compliantie tussen beide methoden na 1 en 6 maanden, en histologisch onderzoek liet ook geen grote verschillen zien. Squameuze differentiatie van het urotheel was 1 maand na reconstructie aanwezig aan de grenzen tussen natief en geregenereerd weefsel in de zieke blazen. Dit suggereert dat urotheliale veranderingen in geregenereerd weefsel van zieke blazen op vroege tijdstippen aanwezig zijn. Na 6 maanden was het urotheel van het geregenereerde weefsel normaal, wat een reversibel effect impliceert.
Regeneratie van blaasweefsel met een zeer poreuze collageen scaffold is mogelijk in een ziek diermodel. Regeneratie van blaasweefsel in het diermodel voor blaasextrofie was vergelijkbaar met regeneratie in gezonde blaas, resulterend in weefsel van goede kwaliteit.

Hoofdstuk 4
Groeifactoren zijn betrokken bij proliferatie, migratie en differentiatie van verschillende celtypes. Heparine kan gebonden worden aan collageen scaffolds, wat de binding van sommige groeifactoren aan de scaffold verbetert en proteolytische afbraak van groeifactoren vermindert, wat resulteert in een systeem met vertraagde afgifte. 'Vascular endothelial growth factor 165' (VEGF165) is een belangrijke factor bij angiogenese. De combinatie met 'fibroblast growth factor 2' (FGF2) verbetert zowel bloedvatvorming als bloedvatrijping. Deze groeifactoren zijn gekoppeld aan collageen scaffolds geladen met heparine, in combinatie met 'heparin-binding epidermal growth factor' (HB-EGF), wat betrokken is bij urotheel regeneratie.
In hoofdstuk 4 werd het effect van de toevoeging van de groeifactoren VEGF165, FGF2 en HB-EGF aan een collageen-heparine scaffold op regeneratie en functionaliteit van blaasweefsel onderzocht. De scaffold werd aangebracht in het blaasextrofie model (COLGF-groep), en de resultaten werden vergeleken met die van een scaffold zonder groeifactoren in hetzelfde model (COL-groep), en reconstructie zonder het gebruik van een scaffold (primaire sluiting) (PC-groep). Functionele en histologische evaluaties werden uitgevoerd na 1 en 6 maanden.
Histologisch onderzoek toonde verbeterde ingroei van urotheelcellen in lammeren uit de COLGF-groep. Hierbij werd aaneengesloten, meerlagig en goed

gedifferentieerd urotheel gevonden na 1 maand, terwijl in de COL-groep een onderbroken laag urotheel werd gevonden. Na 1 maand was, vergeleken met de COL-groep, in de COLGF-groep meer angiogenese zichtbaar. Op beide tijdstippen was in de COLGF-groep de ingroei van gladde spiercellen verbeterd. De verbetering van regeneratie van blaasweefsel leidde niet tot een statistisch significante toename van de urodynamische resultaten.
Een collageen scaffold verrijkt met de groeifactoren VEGF165, FGF2 en HB-EGF verbeterde weefselregeneratie in een groot diermodel voor ziek blaasweefsel. Dit resulteerde in weefsel van goede kwaliteit in alle lagen van de blaas.

Hoofdstuk 5
De huidige standaard urinedeviatie voor patiënten met blaaskanker en sommige pediatrische patiënten met ernstige aangeboren afwijkingen wordt geconstrueerd met ileum. De complicaties die hierbij kunnen optreden zijn vooral gerelateerd aan het gebruik van gastro-intestinale weefsels. Tissue engineering kan de mogelijkheid bieden om alternatieven voor gastro-intestinale weefsels te ontwikkelen. In hoofdstuk 5 hebben we van collageen en polymeer een tubulair construct ontwikkeld, welke in een varkensmodel werd geëvalueerd op zijn toepasbaarheid als urinedeviatie.
Van rundercollageen type I en Vypro® II synthetisch polymeer mesh werd een tubulair construct, 12 cm lang en 15 mm in diameter, geconstrueerd. Het construct werd gesteriliseerd, bezaaid met primaire varkensblaas urotheelcellen of gebruikt zonder cellen, geanastomoseerd met de rechter ureter en geïmplanteerd als een incontinent urostoma in 10 vrouwelijke 'Landras' varkens. Na 1 maand werd het construct geëvalueerd middels functioneel en histologisch onderzoek.
De overleving was 80%, met 1 gerelateerd (volledige stenose van het stoma-uiteinde) en 1 niet-gerelateerd overlijden. Het stoma-uiteinde werd stenotisch bij alle dieren. Na 1 maand was het collageen geresorbeerd en was er een retroperitoneale buis gevormd die 40 cm H_2O druk kon weerstaan. In 5 varkens fungeerde de buis als een urostoma; loopograms lieten bij 3 varkens een stenose bij de ureterale anastomose zien, waarvan er 2 lekkage vertoonden. In 4 varkens werd de polymeer mesh in het lumen van de buizen gevonden, wat niet was afgebroken noch opgenomen in het omliggende weefsel. Bij alle varkens werd rechtszijdig hydroureteronefrose gezien. Histologisch onderzoek toonde een matige immuunrespons en angiogenese. Urotheelcellen waren schaars in het lumen van het construct; wel werd urotheel-bedekking gezien bij de ureteranastomose. De polymeer mesh veroorzaakte fibroblast-depositie en weefselcontractie. Er werden geen grote verschillen waargenomen tussen cellulaire en acellulaire constructen.
Het tubulaire construct vormde een retroperitoneale buis die in de meeste gevallen fungeerde als urostoma. Verbeteringen van deze techniek zijn nodig voordat klinische toepassing mogelijk is, bijvoorbeeld door het gebruik van een afbreekbaar polymeer met verbeterde biocompatibiliteit, en everteren van het stoma-uiteinde

om stenose te voorkomen. Verbeterde grote tubulaire constructen zouden een alternatief voor gastro-intestinaal weefsel kunnen worden bij het construeren van een urinedeviatie.

Hoofdstuk 6
Primaire sluiting van ernstige congenitale buikwanddefecten kan worden bemoeilijkt door hypoplasticiteit van de buikholte en het vergrote volume van de darm als gevolg van oedeem en de vorming van een fibreuze schil. In een foetaal schapenmodel voor gastroschizis werd regeneratie van de buikwand met behulp van een dubbellaags collageen scaffold geëvalueerd en werd de beschermende werking op de darmen bestudeerd.
Bij 14 foetale lammeren werd bij 79 dagen dracht een gastroschizis gecreëerd door de buikwand te openen en de darmen buiten de buikholte te brengen. In 1 groep bleven de darmen onbedekt, in een tweede groep werden de darmen herplaatst in de buikholte en werd het defect gesloten door het inhechten van een collageen scaffold in de buikwand. Bij 140 dagen dracht werd een keizersnede uitgevoerd, en macroscopisch en histologisch onderzoek verricht.
Elf lammeren (79%) werden levend geboren. Bij de 5 lammeren met een gastroschizis waren de darmen coalescent, uitgebreid verkleefd en bedekt met een fibreuze schil. Bij de overige lammeren was de buikwand gesloten, met een stevige verbinding van het nieuwe weefsel met de natieve buikwand. Bij 1 lam was een kleine hernia voelbaar. Er waren weinig of geen verklevingen van de darmen en er was geen fibreuze schil aanwezig. De scaffold was grotendeels afgebroken en vervangen door bindweefsel bestaande uit collageen, fibroblasten, bloedvaten en verspreid liggende spiercellen. Het huidweefsel was meer volgroeid aan de randen ten opzichte van het centrum, met epithelialisatie en adnex-vorming.
Buikwandregeneratie met een collageen scaffold is mogelijk bij foetale lammeren en resulteert bij de geboorte in een gesloten buikwand en geregenereerde huid en bindweefsel met angiogenese en spiercellen. Direct sluiten van de gastroschizis leidde tot een sterke vermindering of tot het voorkomen van darmverklevingen en de vorming van een fibreuze schil.

Hoofdstuk 7
Bij foetussen met gastroschizis worden de darmen beschadigd door toxische stoffen in het vruchtwater en beklemming ter plaatse van het buikwanddefect. Foetale bedekking van de darm die buiten de buikholte ligt, kan verdere secundaire schade aan de darm voorkomen. Het doel van deze studie was om de darm die bij gastroschizis buiten de buikholte ligt, te bedekken met een collageen scaffold om deze te beschermen en om celgroei in de scaffold te induceren, wat zou kunnen leiden tot regeneratie van huid of buikwand.
Bij 12 foetale lammeren werd bij 79 dagen dracht chirurgisch een gastroschizis gecreëerd. Een dubbellaags scaffold van collageen type I werd aan de huid van de

buikwand rond het defect gehecht en bedekte hiermee de darmen. Macroscopisch en histologisch onderzoek werd uitgevoerd na een keizersnede bij 140 dagen dracht. De overleving was 67%. Bij 7 van de 8 overlevende lammeren was de darm bedekt na de geboorte; bij 1 lam was de scaffold gescheurd, resulterend in een gastroschizis. Bij 5 van de 7 lammeren was de darm terug in de buikholte, bij 2 lammeren was de darm nog gedeeltelijk buiten de buikholte. Bij 6 van de 7 lammeren werden slechts minimale verklevingen van de darmlissen gezien, 1 lam had uitgebreide verklevingen, resulterend in een obstructieve ileus. Er werd geen fibreuze schil gezien. De scaffold was vervangen door bindweefsel bestaande uit collageen, fibroblasten en bloedvaten, dat werd bedekt met huidweefsel. Aan de randen was het huidweefsel beter ontwikkeld ten opzichte van het centrum, met epithelialisatie en adnex-vorming.
Prenatale bedekking van de darmen met een collageen scaffold is mogelijk bij foetale lammeren met gastroschizis. Dit leidde tot een sterke afname van beschadiging van de darmen, en de vorming van huid- en bind-weefsel ter vervanging van de scaffold. Het bedekken van de darm, in plaats van herplaatsen van de darm in de buikholte en sluiten van de buikwand zoals in hoofdstuk 6, vereenvoudigt de prenatale operatie. Postnatale buikwandreconstructie kan hierdoor eenvoudiger, of mogelijk zelfs onnodig worden.

Toekomstvisie

Voor een aanzienlijk effect op persoonlijke behandelingsopties op het gebied van 'Regeneratieve Geneeskunde' moeten toekomstige studies gericht zijn op het gebruik van grotere scaffolds, die een klinisch significante vergroting van de blaas kunnen bewerkstelligen. Weefselregeneratie in het centrum van grote scaffolds kan echter belemmerd worden door vertraagde angiogenese in dit gebied. Vanwege gebrekkige aanlevering van zuurstof en voedingsstoffen voor de cellen en onvoldoende afvoer van afvalstoffen, wordt weefselregeneratie belemmerd en kan fibrotisch littekenweefsel of zelfs necrose ontstaan. De hoeveelheid zuurstof, die nodig is voor celoverleving, wordt beperkt doordat de diffusieafstand vanaf het voedende bloedvat slechts ongeveer 150-200 µm bedraagt. Angiogenese moet daarom worden verbeterd in grote scaffolds.
Omentum is rijk aan bloedvaten en wordt rond of over een 'tissue-engineered' construct gewikkeld, om ingroei van bloedvaten in het construct, afkomstig uit het omentum, te verbeteren. We hebben deze techniek gebruikt in de hoofdstukken 3 en 4. Daarnaast kan een gefaseerde reconstructie de weefselregeneratie verbeteren bij het gebruik van een construct bezaaid met autologe cellen. Eerst wordt het construct verpakt in omentum [1] en tijdens een tweede operatie wordt het gehecht aan de blaas. Tussen deze handelingen kan angiogenese in het construct plaatsvinden, waardoor een verbeterd micromilieu voor de gezaaide cellen ontstaat,

nog voordat deze cellen worden blootgesteld aan urine, wat een schadelijk effect op deze cellen kan hebben.
Een andere benadering voor het verbeteren van angiogenese in de scaffolds is het gebruik van groeifactoren, zoals bevestigd in hoofdstuk 4. Grotere scaffolds geladen met groeifactoren, moeten worden getest op het vermogen om blaasweefsel van goede kwaliteit te regenereren in het gehele geïmplanteerde construct. Meer informatie is nodig over de beste combinaties en concentraties van groeifactoren voor elk specifiek weefsel. Van groeifactoren zoals 'platelet-derived growth factor-BB' (PDGF-BB), 'hepatocyte growth factor' (HGF) of 'insulin-like growth factor 1' (IGF-1) is bekend dat ze proliferatie van spiercellen bevorderen. Het gebruik hiervan moet worden onderzocht, mogelijk in combinatie met de groeifactoren uit hoofdstuk 4. Op basis van de testresultaten kan een scaffold met een specifieke combinatie van groeifactoren worden geproduceerd, afgestemd op het te regenereren weefsel.
Mogelijk is de combinatie van groeifactoren én autologe cellen op een scaffold een optie om weefselregeneratie te bevorderen. Autologe cellen worden dan op scaffolds met groeifactoren gezaaid en kunnen gedurende enkele dagen op de scaffold worden gekweekt, waarna het construct wordt geïmplanteerd. Deze theorie moet eerst *in vitro* worden getest, om de invloed van het kweken van cellen op de scaffold op de aanwezige hoeveelheid en activiteit van de opgenomen groeifactoren te evalueren, voordat *in vivo* studies worden uitgevoerd.
Een andere benadering om angiogenese in grote scaffolds te bevorderen wordt momenteel door onze onderzoeksgroep onderzocht. In plaats van één grote scaffold maken we gebruik van een aantal kleinere scaffolds om dezelfde oppervlakte aan geregenereerd weefsel te bereiken. Door de verkorte afstand van de gevasculariseerde rand van het natieve weefsel tot het midden van de scaffold, wordt de tijd verkort totdat dit gebied gevascullariseerd zal zijn.
Schultheiss *et al.* presenteerden een interessante benadering ter verbetering van vascularisatie, die van toepassing kan zijn voor 'tissue engineering' van blaasweefsel [2]. Een dunne darmsegment van een varken werd geïsoleerd en gedecellulariseerd. De matrix werd bezaaid met autologe urotheel- en gladde spier-cellen. Bloedvaten werden bezaaid met endotheliale voorlopercellen door het perfunderen van deze gekweekte cellen. Om de vaten langdurig open te houden werd in latere studies van deze onderzoeksgroep continue perfusie met een peristaltische perfusiepomp toegepast [3]. Hiermee werd een construct gemaakt met een intact netwerk van bloedvaten, welke werden geanastomoseerd tijdens de implantatie in een varkensmodel. Het construct was levensvatbaar en zonder trombusvorming na 3 uur [2]. Een experiment in 1 patiënt toonde de levensvatbaarheid van dit construct gedurende 1 week. In deze studie werden echter alleen endotheelcellen gezaaid in het construct [3].
Turner *et al.* gebruikten een andere 'tissue engineering' techniek voor het verrichten van een blaasaugmentatie [4]. Deze groep isoleerde autologe urotheelcellen uit een biopt, en kweekte deze tot sheets. Tijdens de operatie werd een segment van het

sigmoid geïsoleerd en chirurgisch van epitheel ontdaan. Dit segment werd bedekt met de sheet van urotheel en vervolgens geïmplanteerd in een blaas. De sheet werd bevestigd aan het darmsegment middels een vicryl mesh en een siliconen blaasballon. Dit resulteerde in een construct dat na 3 maanden geheel bekleed was met urotheel, zonder fibrose, slijm, stenen of hergroei van darmepitheel.
Het 'tissue-engineered' tubulaire construct uit hoofdstuk 5 moet worden verbeterd voordat het kan worden gebruikt als een incontinente urinedeviatie. Er moet onderzocht worden of de vascularisatie verbeterd kan worden door het construct in omentum of peritoneum te wikkelen. Tevens moet een afbreekbaar polymeer met verbeterde biocompatibiliteit geëvalueerd worden. Als oplossing voor het tegengaan van stenosering van het stoma-uiteinde zou het everteren van het construct op huidniveau kunnen dienen.

Op dit moment zijn de risico's op complicaties die leiden tot vroeggeboorte de achilleshiel van de foetale chirurgie. Verbeteringen van de techniek, bijvoorbeeld door gebruik van minimaal invasieve behandeling door een laparoscopische benadering van de foetus ('fetoscopy'), zijn noodzakelijk voordat de voordelen van foetale therapie opwegen tegen de risico's. Dit geldt in het bijzonder voor aangeboren afwijkingen die over het algemeen niet dodelijk zijn, zoals gastroschizis. Eerste pogingen om een chirurgisch gecreëerde gastroschizis in een foetaal lam te repareren middels 'fetoscopy' zijn niet gelukt. Het darmvolume was vergroot door de inflammatoire fibrotische schil en opgezwollen darmlissen, wat resulteerde in ernstige hemodynamische problemen bij de foetus wanneer de darm werd teruggebracht in de relatief onderontwikkelde buikholte. Dit kan worden voorkomen door een scaffold te gebruiken bij het sluiten van de buikwand middels 'fetoscopy', zoals beschreven in hoofdstuk 6. Hierbij wordt de buikwand vergroot en zal de intra-abdominale druk minder stijgen dan bij primaire sluiting. Zoals beschreven in hoofdstuk 7, is bedekking van de darmen een optie bij moeilijke gevallen waarbij het herpositioneren van de darm in de buikholte niet mogelijk is. Deze technieken moeten worden getest met behulp van 'fetoscopy'.
Verbetering van het materiaal zou nodig kunnen zijn voor het herstel van foetale buikwanddefecten. In hoofdstuk 6 en 7 vonden we bij 1 lam een gescheurde scaffold en bij 1 lam een kleine hernia, waarschijnlijk veroorzaakt door onvoldoende sterkte van de dubbellaags scaffold. Een mogelijke oplossing is het combineren van de collageen scaffold met een afbreekbaar polymeer.
De toevoeging van groeifactoren aan de scaffold zou ook tot verdere verbetering van buikwand-regeneratie kunnen leiden. In eerder onderzoek hebben we vastgesteld dat de toevoeging van VEGF en FGF2 aan een collageen scaffold een gunstig effect had op regeneratie van foetale huid [5]. De wondcontractie was afgenomen, de angiogenese verbeterd, en er werden minder myofibroblasten gezien. De toevoeging van groeifactoren kan mogelijk leiden tot snellere wondsluiting en wellicht de ingroei van spiercellen verbeteren.

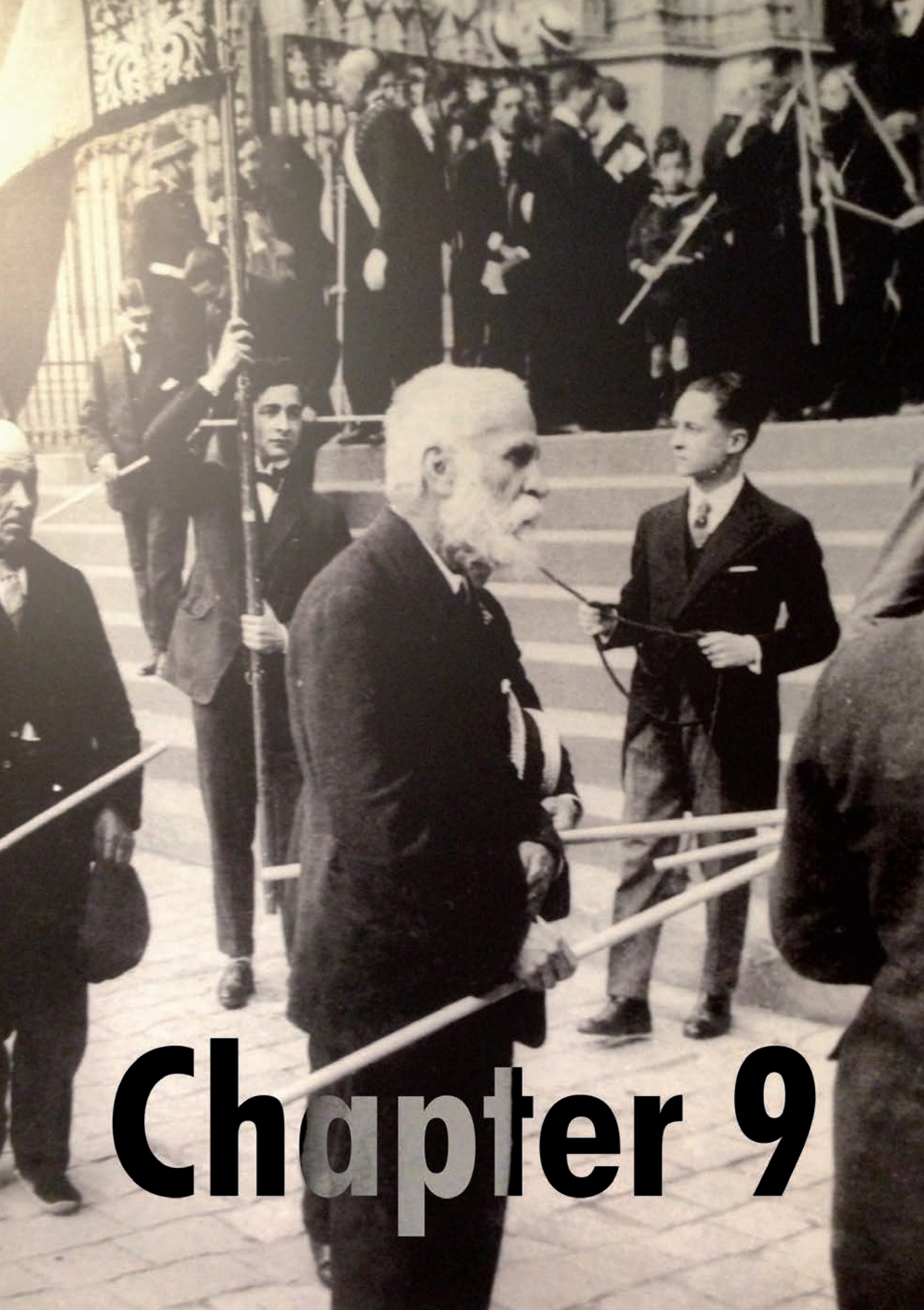
Deze verfijningen moeten uiteindelijk resulteren in producten die in klinische studies kunnen worden getest. Belangrijke zaken moeten echter worden afgewogen bij het gebruik van 'tissue-engineered' producten in klinische studies. Deze afwegingen werden onlangs beschreven door Oerlemans *et al.* [6]. 1) Het 'tissue-engineered' product heeft een veelomvattende complexiteit, aangezien het een zekere variabiliteit en een dynamische interactie met het lichaam heeft, en door de invloed op het omliggende weefsel kan het proces niet ongedaan gemaakt worden. 2) Het testen van het product is moeilijk vanwege het ontbreken van een gouden standaard uit gerandomiseerde klinische trials. 3) De patiënt is een kind met een lange levensverwachting, waardoor de patiënt wordt blootgesteld aan mogelijke lange termijn complicaties, zoals maligne degeneratie. Bovendien moet een 'informed consent' worden verkregen van de ouders, en kan de beslissing gevolgen hebben voor de verre toekomst. De behandeling is zeer complex en heeft andere doelen en risico's dan conventionele chirurgische procedures, waardoor het voor leken moeilijk is een beslissing te maken.

De auteurs doen een voorstel voor een aangepast plan voor het testen van 'tissue-engineered' producten bij kinderen. Diermodellen moeten een optimale weergave zijn van de situatie bij de mens. Daarom moeten diermodellen worden gebruikt of ontwikkeld die de 'zieke' situatie bij de mens nauwgezet nabootsen. Het uitvoeren van een systematische review is een 'evidence-based tool' om het meest geschikte diermodel te identificeren [7,8].

De eerste stap na de dierproeven zijn kleinschalige expert case series met werkelijke patiënten. Voor deze stap moeten potentiële risico's worden geminimaliseerd en voordelen worden gemaximaliseerd. Producten moeten worden geproduceerd volgens 'Good Manufacturing Practice' (GMP) richtlijnen, in GMP-erkende voorzieningen (bijvoorbeeld 'clean rooms') onder gestandaardiseerde omstandigheden en met gecontroleerde sterilisatie-methoden. Vervolgens moeten studies worden uitgevoerd volgens 'Good Clinical Practice' richtlijnen voor klinische studies. Zowel de productie als het testen van de producten moet worden uitgevoerd volgens de 'European Medicines Agency' (EMA) regelgeving van de Europese Unie voor 'Geavanceerde therapieën': 'tissue-engineered producten' of 'gecombineerde geneesmiddelen van geavanceerde therapie' [9]. 'Informed consent' dient te bestaan uit een begrijpelijke uitleg van mogelijke voordelen en risico's op zowel korte als lange termijn.

Tijdens de volgende stap wordt het product geëvalueerd op grotere schaal, in verschillende teams van verschillende expertisecentra en op basis van een uniform protocol. Vervolgens moeten grote klinische studies de superioriteit van het product bewijzen ten opzichte van de conventionele behandeling.

'Translationele geneeskunde' probeert de vertaling te maken van 'bench to bedside', door het implementeren van in laboratoria ontwikkelde producten in de klinische praktijk. In het EuroSTEC project zijn met steun van de Europese Unie grote inspanningen verricht op dit gebied met betrekking tot 'soft tissue engineering' [10]. Hopelijk kunnen de behandelingstechnieken beschreven in dit proefschrift binnen enkele jaren leiden tot betere behandelingsmogelijkheden voor kinderen met ernstige aangeboren afwijkingen.



Chapter 9

Curriculum Vitae

Luc Roelofs werd geboren op 14 april 1978 te Ewijk. In 1996 behaalde hij zijn Atheneum diploma aan het Dominicus College te Nijmegen. In datzelfde jaar werd gestart met de studie geneeskunde aan de Katholieke Universiteit Nijmegen.
In 2003 behaalde hij zijn artsexamen. Aansluitend was hij werkzaam als arts-onderzoeker op de afdeling Urologie van het UMC St Radboud te Nijmegen voor zijn promotieonderzoek (begeleider Prof. dr. W.F.J. Feitz). Hierbij maakte hij deel uit van de werkgroep "Foetale chirurgie en tissue engineering", een samenwerkingsverband tussen de afdelingen Urologie, Verloskunde en Gynaecologie, Kinderchirurgie en Biochemie. In 2005 was hij 4 maanden werkzaam als AGNIO Urologie aan het Rijnstate Ziekenhuis te Arnhem. Februari 2007 kreeg hij een AGIKO-stipendium van ZonMw en het UMC St Radboud voor zijn promotieonderzoek. Tevens maakte het project deel uit van het EuroSTEC project (European project on Soft Tissue Engineering for Congenital birth defects in children) gefinancierd door de Europese Unie. De studies hebben geleid tot meerdere presentaties op nationale (NVU) en internationale (EAU, ESPU) congressen en de publicaties beschreven in dit proefschrift. Voor zijn presentaties kreeg hij onder andere de Vlietstraprijs van de NVU in 2004 en meerdere buitenlandse prijzen.
Maart 2007 startte hij met de opleiding tot uroloog aan de afdeling Heelkunde van Gelre Ziekenhuizen locatie Apeldoorn (opleider Dr. W.H. Bouma). Hierna volgde het perifere deel van zijn opleiding tot uroloog in het Rijnstate Ziekenhuis te Arnhem (opleider Dr. P.C. Weijerman) en het academische deel in het UMC St Radboud te Nijmegen (opleider Prof. dr. J.A. Witjes).
Na het voltooien van zijn opleiding tot uroloog heeft hij in 2013 zijn promotietraject afgerond. In 2013 was hij gedurende 3 maanden Fellow Laparoscopische en Endoscopische Urologie in het St Elisabeth Hospitaal te Willemstad Curaçao (begeleiders Dr. J.J. Bade en Dr. W.A. Isa). In 2014 is hij gestart met een fellowship laparoscopische urologie in het Scheper Ziekenhuis te Emmen (begeleider Drs. B.C. Knipscheer).

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Dankwoord

Dit proefschrift was nooit tot stand gekomen zonder de hulp van een aantal mensen. Graag wil ik deze mensen danken voor de hulp bij het opzetten, uitvoeren, evalueren en opschrijven van mijn onderzoek. Daarnaast diegenen die mij op enigerlei andere wijze geholpen hebben gedurende het project.

Professor dr. W.F.J. Feitz, beste Wout. Jij hebt mij aangenomen op dit onderzoeksproject, daarvoor ben ik je zeer dankbaar. Ik heb met veel plezier met je samengewerkt, zoals ook met de hele projectgroep. Door jouw inspirerende begeleiding hebben wij dit project tot een goed einde kunnen brengen. Jouw steun heeft me niet alleen geholpen bij ons onderzoeksproject maar tevens bij mijn opleiding tot uroloog. Ook daarvoor zal ik je altijd dankbaar blijven. Met name jouw altijd positieve houding, je chirurgische kundigheid, je doorzettingsvermogen en natuurlijk het steeds weer klinkende "typen, typen, typen…" zullen me waarschijnlijk voor altijd bijblijven.

Professor dr. R.M.H. Wijnen, beste René. Als lid van het AHOPAMAK gezelschap ben jij vanaf het begin van ons project als begeleider bij het onderzoek betrokken geweest. Ook jou wil ik danken voor je hulp bij de opzet van het onderzoek, de uitvoer en begeleiding bij de dieroperaties en de analyse en verwerking van de resultaten. Jouw opgewekte houding en je humor heeft voor een zeer prettige samenwerkingssfeer gezorgd, en ook mij geïnspireerd tijdens mijn onderzoek. Ik wens je veel succes in het Erasmus MC als hoofd van de afdeling kinderchirurgie.

Mijn opleiders, Prof. dr. J.A. Witjes, Dr. P.C. Weijerman en Dr. W.H. Bouma, en de overige stafleden van de afdeling urologie van het Radboudumc Nijmegen en het Rijnstate Ziekenhuis Arnhem, en de afdeling chirurgie van het Gelre Ziekenhuis Apeldoorn, wil ik danken voor de begeleiding tijdens mijn opleiding tot uroloog.

Dr. T.H. van Kuppevelt en Dr. ir. W.F. Daamen, beste Toin en Willeke. Jullie maakten een zeer belangrijk deel uit van mijn onderzoek, jullie ontwierpen, produceerden en verbeterden de 'matjes' voor mijn onderzoek. Als 'mattenmakers' waren jullie dus onmisbaar. Tevens wil ik hiervoor Herman van Moerkerk bedanken, die ons helaas ontvallen is. Daarnaast kon ik altijd bij jullie terecht voor vragen over de lastige bereidingswijze met moeilijke chemische termen, waarvan ik als clinicus geen kaas gegeten had… Ook met jullie kritische correcties van mijn manuscripten hebben jullie een grote bijdrage geleverd aan dit proefschrift.

Dr. P.J. Geutjes en D.M. Tiemessen, beste Paul en Dorien. Zonder jullie hulp was dit proefschrift nooit af gekomen. Wat hebben jullie veel werk voor me verzet. Paul, jij bent vanaf 2007 bij het project betrokken en hebt jezelf bijna onmisbaar gemaakt. Helaas koos je voor een andere carrière, maar ook daarin zul je zeker succesvol worden. Bedankt voor al het werk aan dit project, voor onze discussies en de lol die we hebben gehad. Dorien, bedankt voor al het 'lab-werk' wat je voor me verricht hebt. Ik weet niet hoeveel coupes je ondertussen voor me gekleurd hebt, maar het zijn er in ieder geval bakken vol. Ook met jou heb ik veel gelachen, zowel tijdens het werk, als erna in de 'Aesculaaf' of in 'St. Anneke'.

De leden van AHOPAMAK, onze projectgroep 'Foetale chirurgie en Tissue Engineering'. Beste Jane, wat hadden we gemoeten zonder jouw ervaring met de (foetale) chirurgie bij schapen? Je hebt onze groep de fijne kneepjes van het opereren op schapen en het opereren op een schapen foetus bijgebracht. Daarnaast heb je me in contact gebracht met Prof. dr. Feitz, waarmee dus eigenlijk dit hele proefschrift begonnen is.
Dr. A.J. Eggink, beste Alex, je was mijn mede-promovendus op ons foetale chirurgie project. Ik heb ik altijd bijzonder plezierig met je samengewerkt en wens je veel succes in de stad waar je 'roots' liggen, met die volgens jou zo prachtige skyline.
Prof. dr. P.P. van den Berg, beste Paul. Ook jij stond aan de wieg van de AHOPAMAK groep. Zonder jou was dit project er niet geweest, hartelijk dank voor mijn aanstelling op het project en de gezellige momenten, onder andere tijdens de AHOPAMAK meeting op locatie.
Dr. B.B.M. Kortmann en Drs. R.P.E. de Gier, beste Barbara en Robert. Dank voor jullie hulp bij de operaties en adviezen bij het schrijven van de artikelen, en de enerverende tijd tijdens de ESPU congressen.

Dr. E. Oosterwijk, beste Egbert. Hartelijk dank voor je begeleiding en met name je hulp bij het schrijven van mijn artikelen. Met enkele vragen of suggesties wist jij altijd weer licht in de duisternis te scheppen tijdens het schrijven van een discussie.
Prof. dr. Schalken, beste Jack. Jou en alle medewerkers van het lab experimentele urologie wil ik danken voor de mogelijkheid op het lab mijn werk te verrichten, en de ondersteuning daarbij. Succes met jullie toekomstig onderzoek, wat hopelijk nog meer hoogstaand wetenschappelijk werk op urologisch gebied gaat opleveren.

Dr. C.A. Hulsbergen-van de Kaa, beste Christina. Hartelijk dank voor je beoordeling van de histologische coupes van onze eerste studies. Eindelijk begin ik te begrijpen wat ik zie door die microscoop.

De manuscriptcommissie, Prof. dr. M.H. Wijnen, Prof. dr. S.J. Bergé, Prof. dr. M. Ritskes-Hoitinga, wil ik danken voor het beoordelen van mijn manuscript.

De medewerkers van het CDL wil ik bedanken voor de uitstekende begeleiding bij de proefdierexperimenten, met name Alex, Jeroen, Maikel, Conrad, Wilma, Conny en Ton. Met jullie hebben we altijd prettig samengewerkt in een ontspannen sfeer. Alex, nog veel succes met je glanzende voetbalcarrière.

Ko Hagoort wil ik danken voor de stijladviezen tijdens het schrijven van mijn manuscripten.

Mijn collega arts-onderzoekers: Martijn, Kees, Farida, Paula, Jody, Kim, Kamiel, Floor, en later het komische duo Sybren en Tom, de muggen Maurits en Guido, Henk en Katrien van de matrixbiochemie, Jos, Gisele, Harm, Dick, Ruben, Rianne, Alexander, Max, Stijn, Boy, wil ik danken voor de ondersteuning en de gezelligheid op de werkvloer, in St. Anneke, de Aesculaaf, tijdens de uroski en de EAU congressen. Iedereen veel succes tijdens de opleiding tot uroloog en tijdens de carrière als uroloog of in een ander medisch vakgebied.

Mijn vriendengroep de 'DD-ers' uit Ewijk, met name Teun en Hans, mijn paranimfen, Jeroen, Sjors, Joost, Pieter, Paul, Theodor en Erik wil ik bedanken voor de broodnodige ontspanning naast het werk aan mijn proefschrift en de opleiding tot uroloog, evenals de leden van het 'Disputa di Cazzo Grande', Frank, Stijn, Martijn, Joris, Johan, Olivier. Al waren de wintersportvakanties en de avonden stappen niet altijd even ontspannend… Ondanks dat iedereen het steeds drukker krijgt hoop ik dat we elkaar zo veel mogelijk blijven zien.
En natuurlijk mijn voetbalteam Ewijk 6, waarmee we elk jaar weer op jacht gaan naar het kampioenschap en elk weekend de meest hoogstaande wedstrijden spelen voor de eer van de v.v. Ewijk.

Iris en Bernard, wat fijn is het altijd weer om bij jullie te zijn, samen met Frank en Fleur! En hoe heerlijk normaal en 'down to earth' is het altijd met jullie, zo lekker relativerend. Ik hoop nu wat extra tijd te krijgen om jullie vaker te zien!

En als laatste Pa en Ma. We spreken onze gevoelens niet vaak uit, maar wat ben ik blij met jullie als mijn ouders. Dankzij jullie heb ik de mogelijkheid gehad te kunnen studeren, daarin hebben jullie me altijd gesteund. Nog altijd staan jullie voor me klaar om mij ergens mee te helpen. Daar ben ik jullie ontzettend dankbaar voor. Ik hoop dat we nog heel lang in goede gezondheid met z'n allen bij elkaar kunnen zijn.

Dit proefschrift werd mede mogelijk gemaakt door:

ISBN: 978-94-6108-646-4